

AN EXPLORATION OF POSSIBLE MECHANISMS
UNDERLYING THE BENEFICIAL EFFECTS OF
ENRICHED REHABILITATION ON POST-STROKE
RECOVERY OF FUNCTION

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**An exploration of possible mechanisms underlying the beneficial effects of enriched
rehabilitation on post-stroke recovery of function**

by

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Abstract

A combination of enriched environment and daily reaching therapy (i.e. enriched rehabilitation) enhances functional recovery following focal ischemia in rats. However, the mechanisms contributing to recovery, and potentially critical influence of timing of rehabilitation, remain unclear.

Enriched rehabilitation is especially effective when initiated early, however the mechanisms underlying this critical time window have not been determined. Chapter 2 examined the effects of early rehabilitation on neuronal activation (FosB/ Δ FosB), delayed cell death (Fluoro Jade C) and inflammation (ED-1) following endothelin-1 induced focal ischemia. Enriched rehabilitation increased the activity of perilesional cortex in the early post-stroke treatment period. This increased activity likely contributes to the neuroplastic changes and functional recovery observed with extended periods of rehabilitation. Importantly, enriched environment alone did not lead to enhanced activity suggesting that task-specific rehabilitation is necessary to promote maximal recovery. Changes in FosB/ Δ FosB expression provide a powerful approach for detecting use-dependent recovery of function following stroke or brain injury.

Most recovery tends to occur early after stroke, while improvement in the chronic phase is limited. Chapter 3 examined the effects of periodic returns to therapy in the chronic phase of recovery following focal ischemia. Ischemic rats were treated for nine weeks

with enriched rehabilitation, then returned for two periods of intensive “tune-up” therapy. Functional outcome was assessed throughout the treatment period, and neuroplastic changes were measured at the end of the study using Golgi-Cox analysis. While early rehabilitation provided enhanced recovery in both reaching and foot placement ability, there was little benefit of more chronic phases of treatment.

Chapter 4 shows that enriched environment has differential effects on recovery of skilled and unskilled motor functions after focal ischemia. Ischemic rats exposed to longer daily periods of enrichment, in combination with reach therapy, showed greater improvement in reaching ability compared to those receiving less exposure to enrichment. However, all groups showed similar recovery in foot placement and forelimb use, suggesting that even short durations of enriched environment are sufficient to promote recovery of unskilled motor functions. The synergistic effect of enrichment and task-specific therapy provides further evidence that stimulating environments should be incorporated into rehabilitation programs.

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List of Abbreviations

AP - anterioposterior

CIMT – constraint induced movement therapy

DV - dorsoventral

EE – enriched environment

ER – enriched rehabilitation

ER+TU – enriched rehabilitation + tune-up therapy

ER3 – enriched rehabilitation including 3 hours enriched environment per day

ER6 – enriched rehabilitation including 6 hours enriched environment per day

ET-1 – endothelin-1

ER20 – enriched rehabilitation including 20 hours enriched environment per day

ICMS – intracortical microstimulation

MCA – middle cerebral artery

MCAO – middle cerebral artery occlusion

ML - mediolateral

NDS – neurological deficit score

SEM – standard error of the mean

ST – standard housing

ST+TU – standard housing + tune up therapy

Sx - surgery

tPA – tissue plasminogen activator

TU – tune-up therapy

Co-Authorship Statement

Dr Corbett and I were responsible for the concept and experimental design of all studies included in this thesis.

Chapter 2

I was primarily responsible for the concept and design of this study, with significant advice from Dr Corbett and other members of our research team. I performed all practical aspects of this study including testing, surgery, treatment and histology. I carried out all statistical analyses and prepared the manuscript (with assistance from Dr. Corbett).

Chapter 3

I was the primary contributor to all *in vivo* aspects of this study (behavioural training and testing, surgery, enriched rehabilitation treatment and sacrifice). Co-authors Suzanne Evans (research technician), Garry Chernenko (research technician) and Dr. Hana Mala (visiting student from the University of Copenhagen, Denmark) processed the tissue and carried out Golgi-Cox analysis. I performed all statistical analyses and prepared the manuscript (with assistance from Dr. Corbett).

Chapter 4

A portion of the *in vivo* aspects of this study (behavioural training and testing; enriched rehabilitation treatment; and behavioural analysis) was carried out by summer undergraduate student, Pamela Doran. I performed the remainder of these tasks, as well as all surgeries and sacrifices. I carried out all statistical analyses and prepared the manuscript (with assistance from Dr. Corbett).

Chapter 1 Introduction

1.1 General Rationale

Stroke is a leading cause of death and disability worldwide, striking approximately 40,000 – 50,000 Canadians each year. With an aging population and the prevalence of unhealthy lifestyles, it is expected that the impact of stroke will continue to increase in coming years. In fact, the Heart and Stroke Foundation of Canada [2003] has estimated that the incidence of stroke may double by 2050. It is essential that more research focus on finding effective ways to prevent and treat stroke.

A significant number of stroke survivors are left with a permanent physical impairment (Duncan et al., 1992; Nakayama et al., 1994) which impacts on their quality of life and ability to perform activities of daily living. Due in part to a lack of neuroprotective therapies and acute interventions, rehabilitation is the only treatment option for most stroke patients. More efforts to understand the recovery process and optimize rehabilitative approaches must be made in both clinical and basic science research.

1.2 Pathophysiology of Stroke

There are two main types of stroke: hemorrhagic and ischemic. Hemorrhagic stroke occurs due to a rupture of a blood vessel supplying the brain or overlying structures. Ischemic stroke is due to a blockage in one of these blood vessels, leading to a severe reduction or complete lack of blood flow to the supplied tissue. Ischemia accounts for

~80% of stroke incidence, and the research included in this thesis has been carried out using a model of ischemic stroke.

An interruption of blood supply to brain tissue leads to a lack of oxygen and glucose in the affected region. Cells in this region die rapidly due to anoxia and necrotic processes, forming the ischemic core (Dirnagl et al., 1999; Murphy et al., 2008). The nearly immediate energy depletion results in permanent depolarization of neurons and the release of neurotransmitters, including the excitatory transmitter glutamate. Activation of glutamate receptors in nearby neurons results in further depolarization – a process known as “spreading depolarization”. The subsequent failure of energy-dependent processes such as calcium buffering and ion channel gating leads to ionic imbalance. A significant increase in intracellular calcium triggers a number of pathways and releases more glutamate and radical oxidative species, causing widespread cell death. Figure 1.1 summarizes the ischemic cascade leading to cell death following stroke.

While cells in the ischemic core are damaged irreversibly and die quickly, cells in the surrounding tissue remain vulnerable to excitotoxic, apoptotic or inflammation-mediated cell death. This region is termed the penumbra or peri-infarct zone and, due to the more delayed mechanisms of death and injury taking place there, offers potential targets for neuroprotection and salvage (De Keyser et al., 1999).

1.3 Animal models of focal ischemia

There are two general animal models of ischemia: 1) Global ischemia, which produces a transient interruption of blood supply to the entire forebrain, selective damage to CA1/CA2 neuronal populations of the hippocampus and subtle cognitive impairments. This is similar to the ischemic consequences of cardiac arrest in humans. 2) Focal ischemia results from the occlusion of major vessels, such as the middle cerebral artery (MCA) which supplies blood to much of the dorsolateral cortex and striatum. This reduction in cerebral blood flow frequently produces profound sensorimotor impairments.

In order to study motor recovery after stroke, it is important to use models of focal ischemia that adequately reflect both the pathophysiology and physical impairments of clinical stroke (Carmichael, 2005). While models of ischemia have been developed in several species, rats have been used most extensively for studies of functional impairment and recovery (Kleim et al., 2007). Firstly, experiments using rats are substantially more practical and cost-effective than those using primate models of ischemia. Secondly, rats have brain structure and cerebrovasculature that approximates that of humans, allowing for development of models that produce similar neurological injury and symptoms (Cenci et al., 2002). Thirdly, rats exhibit a number of motor behaviours that more closely resemble those of humans than do most other non-primate species, and these behaviours have been well studied and characterized. Most importantly, rats are prehensile and show similar patterns of limb movement and skilled reaching when compared to humans (Whishaw and Pellis, 1990; Whishaw et al., 1992; Sacrey et al., 2009). Finally, following

focal ischemia or brain injury rats exhibit perturbations of these reaching movements (Whishaw et al., 1991; Whishaw et al., 1993; Gharbawie et al., 2006; Clarke et al., 2007a; Alaverdashvili and Whishaw, 2008) that are reflective of the upper extremity impairments shown by many stroke survivors. These similarities make rat models particularly useful to study patterns of recovery following stroke and the effects of interventions aimed at improving functional outcome.

1.3.1 Middle cerebral artery occlusion

Middle cerebral artery occlusion (MCAO) has been the most utilized model of focal ischemia because it reflects the most common form of clinical stroke. A number of techniques to occlude the MCA have been developed, each producing slightly different patterns of injury and impairment. The most widely used technique involves inserting an intraluminal suture into the artery, occluding blood flow (Longa et al., 1989). The suture may be left in place either permanently or for a specific period of time (typically 60-120 minutes). Other techniques involve cauterizing (i.e. permanent occlusion) or clipping the distal portion of the artery (Tamura et al., 1981; Buchan et al., 1992), or creating an embolism via the injection of microspheres (Gerriets et al., 2003) or autologous blood clots into the artery (Zhang et al., 1997).

Regardless of the technique used and/or the duration of occlusion, MCAO tends to produce variable injury to both the cortex and subcortical structures (striatum, thalamus and hypothalamus) (Kanemitsu et al., 2002; Dittmar et al., 2003; Carmichael, 2005),

unless long durations (e.g. 90-120 minutes) of ischemia are employed. This results in very large infarcts, frequently creating damage to the hypothalamus, which are not representative of most forms of human stroke (Carmichael, 2005). Further, MCAO typically tends to spare the forelimb motor cortex (Windle et al., 2006), which is often affected in clinical stroke. This may have important consequences for experiments assessing functional recovery, and consideration should be given to the appropriateness of this model when designing such studies.

Other concerns with this model include the very immediate reperfusion that occurs following a transient occlusion, especially after removal of an intraluminal suture. This rapid reperfusion does not accurately reflect the more gradual reperfusion that generally takes place in clinical stroke (Carmichael, 2005) (except of course following administration of tPA – see Section 1.4.1 for more information) and may result in markedly different inflammation and oxidative stress cascades. Additionally, several techniques of distal MCAO require experimenters to access the artery via the temporal portion of the skull, resulting in injury to the muscles of mastication (Kleim et al., 2007). This often causes severe eating problems for the animals, requiring extra medical care and potentially exacerbating some of the post-ischemic symptoms of the model.

1.3.2 Endothelin-1

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide that can be used to transiently occlude blood vessels when applied directly to or nearby their surface (Yanagisawa et al.,

1988; Fuxe et al., 1997). ET-1 can be injected stereotaxically adjacent to the middle cerebral artery to replicate MCAO models (Sharkey et al., 1993; Sharkey and Butcher, 1995; Biernaskie et al., 2001), or it can be injected so as to target specific brain regions (Windle et al., 2006). This model is more similar to human stroke resulting from embolism or thrombosis (Carmichael, 2005) because reperfusion occurs gradually over the course of hours (Macrae et al., 1993; Biernaskie et al., 2001; Windle et al., 2006).

ET-1 applied to the forelimb motor cortex and dorsolateral striatum produces moderate sized ischemic injury and persistent motor impairments in rats. Compared to ET-1 induced MCAO, this model has a higher success rate, resulting in less mortality and more consistent injury (Windle et al., 2006). The targeted nature of the infarct and the resulting forelimb-specific sensorimotor deficits make this model a good choice for experiments studying functional recovery. Hence, this model was used for all experiments included in this thesis.

1.3.3 Photothrombosis

An alternative method of producing small, circumscribed ischemic lesions in the cortex uses the photosensitive dye Rose Bengal. The dye is injected intravenously or intraperitoneally, and a light source is placed over the cortical area of interest (in rats, the skull is either thinned or a small craniotomy is performed to expose the area.). The irradiation of the dye in underlying vessels produces photooxidation that in turn causes endothelial damage, platelet activation and occlusion (Watson et al., 1985).

While photothrombosis produces small, circumscribed lesions with less variability than most other models it also has a number of disadvantages. Importantly, ischemic lesions produced using this method have little or no reperfusion. Additionally, a lack of penumbral tissue makes this a poor choice for neuroprotection interventions that would target this region (Carmichael, 2005).

1.3.4 Devascularization

Small cortical infarcts can be produced using devascularization, either by permanently coagulating cerebral arteries (Kleim et al., 2003a) or by stripping away pial vessels (Gonzalez and Kolb, 2003). While devascularization does create small circumscribed lesions with less variability than most other models, it does have several major downfalls. Firstly, there is mechanical damage (i.e. trauma and hemorrhage) to the cortex using these methods (Del Bigio et al., 1996; Kleim et al., 2007). Secondly, devascularization does not allow any reperfusion and does not create a penumbral region surrounding the ischemic core. In these ways, it does not accurately reflect the mechanisms of clinical stroke and limits its usefulness for a variety of experimental designs including neuroprotection and rehabilitation (Carmichael, 2005).

1.3.5 Non-ischemic models of injury

Several models of “stroke-like” brain injury are used in the literature that do not actually involve ischemia and are thus not reflective of cerebral ischemia. While these injury models have proved useful in delineating some of the mechanisms involved in recovery and neuroplasticity after brain injury, careful consideration is warranted when interpreting the results of these experiments.

Electrolytic lesions have been used to model small cortical infarcts. While these lesions can be very targeted and infarct size relatively well controlled, the mechanisms of tissue damage and cell death are considerably different than occurs following ischemia and there is no penumbral region surrounding the injury. Aspiration lesions involve mechanically removing small sections of cortical tissue, often motor cortex. Importantly, these lesions produce instantaneous and complete destruction of the removed tissue, while cerebral ischemia produces damage over the course of hours and days. The lack of a penumbral region, the presence of mechanical damage and an entirely different inflammatory response compared to ischemic injury are limiting factors in applying many of the findings of experiments using these models to stroke (Uryu et al., 2001; Gonzalez and Kolb, 2003).

1.4 Current treatment approaches for acute ischemic stroke

1.4.1 Tissue plasminogen activator (tPA)

Currently, the only approved pharmacological intervention for acute stroke is tissue plasminogen activator (tPA), a proteolytic enzyme that breaks down blood clots and restores blood flow following ischemic stroke. A significant limiting factor of this treatment is that it must be administered within 3 to 4.5 hours of stroke onset in order to be effective (Albers et al., 2002) (although this time window is slightly longer if administered by catheter directly to the site of occlusion). Secondly, the stroke must be confirmed as ischemic using a CT scan because there is enhanced risk if used in the case of hemorrhagic stroke. Many small health facilities may not have the equipment required, and the wait time to receive a scan can be detrimental due to the short treatment time window. Together, these restrictions on the use of tPA have proved challenging and only a small percentage (3-11%) of patients are able to receive it (Barber et al., 2001; Katzan et al., 2004; Laloux et al., 2007; Allen et al., 2009).

1.4.2 Neuroprotection

A vast amount of research effort in the past several decades has focused on finding pharmacological agents that can disrupt cell death processes following stroke and effectively save dying tissue (i.e. neuroprotection). These agents, aimed at reducing the amount of damage following stroke, have targeted various biochemical events but mainly

those involved in the eventual excitotoxic death of neurons. For example, scores of drugs have attempted to reduce the deleterious effects of glutamate, either by preventing its release or blocking its receptors. To date, all agents have failed in clinical trials, and many more have never made it to that stage (Lo et al., 2003; O'Collins et al., 2006). While neuroprotection remains an important avenue of research, it is essential that pre-clinical studies be better designed in order to identify more ineffective candidates before they are unduly placed into long and expensive clinical trials (Corbett and Nurse, 1998; De Keyser et al., 1999).

1.4.3 Hypothermia

The neuroprotective benefits of hypothermia following cerebral ischemia have been demonstrated in animal models for nearly two decades, and are now being translated into clinical application. Prolonged lowering of core body temperature has been found to decrease cell death and improve functional outcome following experimental ischemia (Colbourne and Corbett, 1994; Corbett et al., 1997; Colbourne et al., 2000; Dong et al., 2001) and intracerebral hemorrhage (MacLellan et al., 2004). Research suggests that hypothermia acts as a potent neuroprotectant because it effectively targets several key steps in the processes involved in cell death and tissue damage following ischemia. For example, hypothermia lowers metabolic rate, attenuates inflammation, reduces apoptosis, decreases oxidative stress, modulates Ca^{2+} signaling, and increases Mg^{2+} levels which in turn blocks glutamate receptors (Colbourne et al., 1997; Corbett and Thornhill, 2000).

Despite the fact that numerous clinical studies now show that hypothermia can reduce mortality and improve outcome following stroke and traumatic brain injury (Lyden et al., 2006; Hemmen and Lyden, 2009), it remains rarely used. This may be in part due to the potential complications of hypothermia (e.g. pneumonia), but is likely more attributable to the lack of clinical guidelines (Lyden et al., 2006). Official recommendations regarding how and when to use hypothermic treatment must be developed, and appropriate training provided to physicians and staff. The increasing use of hypothermia to treat acute cardiac arrest may lead to better clinical understanding and eventually greater use in acute stroke.

1.5 Motor recovery following stroke

1.5.1 Patterns of motor recovery

Some degree of motor recovery is usually observed following stroke. While the extent and timeline of recovery varies across patients, there are several consistencies that suggest a common pattern. Studies have shown that the majority of spontaneous improvement often takes place in the first weeks and months after ischemic onset, while further recovery is limited during more chronic phases (Duncan, 1994; Duncan et al., 2000; Hendricks et al., 2002). Patients with mild impairments tend to experience recovery earlier and to a greater extent than those with severe impairments (Jorgensen et al., 1995; Kwakkel et al., 2003), however recovery may progress over a more prolonged period following severe stroke (Cramer, 2008). One study of upper limb impairment

following stroke showed that 80% of patients had achieved maximal (though not full) recovery of function just three weeks post-stroke (Nakayama et al., 1994).

It is important to note that not all improvements in outcome as measured clinically are due to “recovery” per se. Compensation, or behavioural adaptation, can result in significant improvement in task performance with the patient exhibiting little in the way of “true recovery”. Recently, Levin and colleagues (2009) attempted to clearly define and differentiate recovery and compensation based on three levels of the International Classification of Health, Function and Disability (ICF): health condition, body structure and function, and activity. Respectively, motor recovery involves reinstatement of original function in the tissue that was lost, restoration of the ability to perform a motor movement in the same way as prior to stroke, and ability to successfully complete a task in the way it is typically completed by a non-disabled person. Compensation, on the other hand, includes the acquisition of a function by neural tissue that it did not have prior to injury, performance of a movement in a new way, and the use of atypical techniques to complete a task. Because most animal and human studies use performance outcome measures to assess “recovery”, true motor recovery and motor compensation are rarely examined separately.

1.5.2 Mechanisms of recovery

The mechanisms underlying “spontaneous” motor recovery are unclear, however evidence supports the involvement of several factors. Firstly, it is plausible that some of

the early recovery observed is due to resolution of diaschisis – a transient depression of metabolism and function in brain regions that are distal from, but connected to, the infarcted tissue (Seitz et al., 1999; Butefisch et al., 2003; Carmichael et al., 2004). Secondly, a significant body of evidence suggests that recovery is mediated by neuroplastic changes that take place in the brain following injury.

1.5.3 Neuroplasticity and functional recovery

1.5.3.1 Neuroplasticity in the intact brain

A large body of evidence has shown that the brain is more capable of experience-driven change than previously thought. The concept of neuroplasticity was first introduced by Ramon y Cajal in 1892 (DeFelipe, 2006), while Donald Hebb later suggested that it was the basis for processes of learning and memory [Hebb, 1949]. However, the importance of neuroplasticity was largely ignored for several decades.

Several seminal studies in the 1980's and early 1990's showed that if a cortical region was deprived of its sensory input, it could later be activated by other (typically somatotopically adjacent) stimuli (Kaas et al., 1983; Merzenich et al., 1984; Donoghue et al., 1990; Garraghty et al., 1991). These were among the first studies illustrating a reallocation of function in cortex in adult brain – where one region can adapt to take over the function of nearby regions. Over the subsequent three decades, research has clearly shown that the brain is continuously changing and remodeling in response to experience.

Importantly, Nudo and colleagues (1996b) showed that motor cortex representation of the hand and digits can be increased by motor training in adult monkeys. Similar increases in distal forepaw representation, along with synaptogenesis, have been observed in the motor cortex of rats trained to reach for food rewards (Kleim et al., 1998; Kleim et al., 2002). These neuroplastic changes in the intact brain are likely key to the processes that underlie recovery and neural repair following stroke and other types of brain injury.

1.5.3.2 Neuroplasticity following brain injury

Evidence that brain structure and physiology is dynamic and changes in response to experience led researchers to investigate the role of neuroplasticity in motor recovery after injury. We now know that injury sparks a number of neuroplastic responses in the brain, and these changes are intricately involved in neuro-reparative processes and recovery of function. Indeed, evidence suggests that focal brain injury renders the brain more able to change structure and functional organization in response to experience.

Starting days after an ischemic cortical injury, neural networks both surrounding and distal from the lesion become hyperexcitable (Buchkremer-Ratzmann et al., 1996; Neumann Haefelin and Witte, 2000). These electrophysiological changes appear to be mediated by an increase in excitatory glutamate receptors and a simultaneous decrease in inhibitory GABA receptors (Neumann-Haefelin et al., 1998). It is likely that this enhanced excitability of neural networks, which is an LTP/LDP type process, contributes

by promoting the formation of new connections and cortical “re-wiring” in the aftermath of injury.

Numerous studies have shown an increase in neurotrophic factors in the acute phase following ischemia, including fibroblast growth factor, vascular endothelial growth factor, neuronal growth factor, insulin-like growth factor-1 and brain-derived neurotrophic factor (Lee and Bondy, 1993; Lennmyr et al., 1998; Cramer and Chopp, 2000; Griesbach et al., 2004; Carmichael, 2006). Interestingly, the majority of these factors are produced by astrocytes (Ridet et al., 1997; Chen and Swanson, 2003), suggesting that the reactive gliosis (proliferation of astroglia in and around the lesion) known to occur after stroke may serve a role in regulating and promoting neural repair. This increase in neurotrophic factors is accompanied by an enhanced expression of both growth-promoting and growth-inhibiting genes in the peri-infarct region, further supporting the theory that the post-ischemic milieu is conducive to reparative processes. The time course of activation appears to favour the growth-promoting genes in opposition to growth-inhibiting genes, providing a window of enhanced neuroplasticity (Carmichael et al., 2005; Li and Carmichael, 2006).

Indeed, enhanced neuronal growth has been observed in peri-infarct cortex following ischemic injury. Axonal sprouting takes place in the intact tissue surrounding cortical lesions in rats, with most outgrowth occurring in the first 2-3 weeks post-injury and declining to normal levels thereafter (Stroemer et al., 1995). Similarly, neurons in this

region undergo significant dendritic hypertrophy resulting in increased branching and density (Jones and Schallert, 1992, 1994; Kolb et al., 2001). These processes likely facilitate cortical remodeling and the development of new functional connections with adjacent intact tissue (Dancause et al., 2005). The subsequent pruning of some neurons that has been observed (Jones and Schallert, 1992) might represent the elimination of inappropriate or redundant connections that have been formed, and is likely an important part of the recovery process. Similar neuronal outgrowth takes place in the contralesional cortex (Jones and Schallert, 1992; Napieralski et al., 1996; Jones, 1999; Papadopoulos et al., 2002) and studies suggest that the intact hemisphere is involved in functional recovery and compensation, especially in patients with large infarcts (Cramer and Riley, 2008; Schaechter et al., 2008).

Cortical reorganization also takes place following injury, with the function of lost tissue being at least partially transferred to adjacent regions (Chollet et al., 1991; Cramer et al., 1997; Cao et al., 1998; Feydy et al., 2002; Ward et al., 2003). It is likely that this remodeling of cortical maps is experience-dependent, implicating a role for post-ischemic rehabilitation in the promotion of recovery and neural repair. While functional imaging and intracortical microstimulation studies in animals (Nudo et al., 1996a; Dijkhuizen et al., 2001; Ramanathan et al., 2006) have suggested that the amount of cortical reorganization correlates positively with recovery after cortical injury, evidence from studies in humans is more equivocal (Cramer et al., 2006).

1.6 Rehabilitation

1.6.1 Rehabilitation and motor recovery

For the large percentage of stroke survivors that are left with a chronic physical impairment, rehabilitation is the only treatment option available. While there is ample evidence that rehabilitation can reduce morbidity and enhance motor recovery (Langhorne and Duncan, 2001; Van Peppen et al., 2004), it remains insufficient to provide a full return of function for most patients.

Even with intensive, effective rehabilitation many patients do not make a full recovery and generally reach a “recovery plateau” after a few months of improvement (Page et al., 2004a). There is growing evidence that therapy approaches that include progressive intensity and novel tasks that challenge patients at each stage of recovery may promote some further recovery, and that quality outpatient rehabilitation programs in the chronic phases of stroke do continue to improve outcome (Smith et al., 1999; Whitall et al., 2000; Page et al., 2004b; Rijntjes et al., 2008). The mechanisms of recovery in the late phase are most likely quite different than those of early phase recovery. More research is needed to shed light on these mechanisms and find ways to optimize stroke rehabilitation programs.

1.6.2 Mechanisms of rehabilitation: insights from animal models

The development of animal models of rehabilitation over the past decade has provided great insight into the neural mechanisms of recovery and the complex relationship between rehabilitation and neuroplasticity.

A number of approaches have been taken in developing rehabilitation models in animals. Most models use either exposure to enriched living conditions, motor rehabilitation tasks, or a combination of both. Enriched living environments provide sensory, motor and social stimulation and promote both sensorimotor and cognitive activity. In intact animals, enriched environments have been found to upregulate plasticity-related growth factors, enhance neurogenesis and increase neuron size, dendritic branching and spine density (Comery et al., 1996; Kempermann et al., 1997; Kempermann et al., 1998; Pham et al., 1999; Ickes et al., 2000; Pham et al., 2002; Faherty et al., 2003; Bruel-Jungerman et al., 2005; Leggio et al., 2005). Motor rehabilitation tasks generally target a specific motor impairment (e.g. reaching or walking) and provide regular, repetitive activity to promote use of impaired limbs and bring about functional improvement. Studies have suggested that a combination of enriched environment and task-specific rehabilitation (often referred to as “enriched rehabilitation”) is an effective model of rehabilitation, at least in rats, where functional benefits are seen both in general neurological deficits (e.g. locomotion, forelimb asymmetry) and in impairments of skilled motor function (e.g. skilled reaching ability) (Biernaskie and Corbett, 2001; Biernaskie et al., 2004).

Animal models have revealed a number of key principles that have in turn directed research and policy in the clinical studies. Firstly, animal studies have clearly demonstrated that earlier is better. Ischemic rats exposed to enriched rehabilitation starting at 5 or 14 days after injury recover significantly better than those with treatment delayed for 30 days after injury (Biernaskie et al., 2004). Similarly, reach training that is delayed one month in monkeys with motor cortex lesions is unable to prevent the loss of movement representation in perilesional tissue that is observed when training begins early after injury (Barbay et al., 2006). Secondly, the intensity of rehabilitative activity has been shown to have a direct impact on neuroplasticity and functional recovery. For example, rats trained to reach 60 times per day did not exhibit the increased synaptic density in motor cortex observed in those trained to reach 400 times per day (Kleim et al., 2002; Luke et al., 2004). Similarly, exercise intensity has been found to differentially influence the expression and upregulation of growth factors associated with neuroplasticity following focal ischemia (Ploughman et al., 2007b). Thirdly, animal studies have shown that task specific rehabilitation is essential to promote recovery of complex movements and abilities. Enriched rehabilitation, with a daily reaching component, has been successful at promoting recovery of reaching performance in ischemic rats (Biernaskie and Corbett, 2001; Biernaskie et al., 2004), while enriched environment alone does not (Grabowski et al., 1993). Interestingly, skilled reaching rehabilitation increases the cortical forelimb representation in intact monkeys (Plautz et al., 2000) and rats with motor cortical lesions (Maldonado et al., 2008), while performance of unskilled movement does not.

Importantly, animal models of rehabilitation have shed light on the neural correlates of motor recovery. Following up on their important studies on neuroplasticity in the intact brain, Nudo and colleagues (1996a) retrained monkeys with small lesions in the forelimb motor cortex on a skilled reaching task. While untrained monkeys experienced a decrease in cortical representation of the hand and digits following injury, animals exposed to reaching rehabilitation maintained or showed increases in representation. This training-induced cortical reorganization was such that areas of cortex that were previously associated with motor control of the arm and shoulder took over the function of hand and digit movement. Similar perilesional reorganization has been observed using intracortical microstimulation in rats that were exposed to daily reach training (Kleim et al., 2003a; Conner et al., 2005; Ramanathan et al., 2006).

Rats exposed to rehabilitation also exhibit neuroplastic changes at the cellular/structural level. Biernaskie and Corbett (2001) found that rats exposed to nine weeks of enriched rehabilitation starting 15 days after MCAO showed increased dendritic branching in the contralesional forelimb motor cortex that coincided with functional recovery on tests of locomotion and skilled reaching. Similar contralesional cortical plasticity and functional recovery has been observed in rats with forelimb sensorimotor cortex lesions exposed to “acrobatic” training (traversing an obstacle course) for several weeks (Jones et al., 1999). Importantly, like the functional benefits of enriched rehabilitation, these neuroplastic changes appear to be dependent on the timing of treatment (Biernaskie et al., 2004).

Several studies have shown a positive correlation between the amount of neuroplastic remodeling and the extent of functional recovery following rehabilitation (Ramanathan et al., 2006; Eisner-Janowicz et al., 2008). Interestingly, adjunct therapies that further promote neuroplasticity, such as cortical stimulation, appear to enhance the functional benefits of motor training after brain injury (Adkins-Muir and Jones, 2003; Kleim et al., 2003a; Plautz et al., 2003; Teskey et al., 2003). Conversely, disruption of neuroplastic mechanisms during reach training interrupts functional organization of cortex and impairs motor movement (Kleim et al., 2003b; Luft et al., 2004), and disruption of activity in brain regions thought to be involved in neuroplastic remodeling after rehabilitation has been found to reinstate motor impairments (Biernaskie et al., 2005).

1.6.3 Mechanisms of rehabilitation: insights from the clinical setting

Numerous theories for the effectiveness of post-stroke rehabilitation have been put forward, and it is likely that these mechanisms work in concert to promote functional improvement. One probable benefit of rehabilitation for recovery is the increased strength and tone of muscles that have weakened in the aftermath of stroke. The strengthening of these muscles has been found to correlate directly with improved functional performance (Flansbjer et al., 2008; Lexell and Flansbjer, 2008; Kluding and Gajewski, 2009).

Another potential mechanism is that rehabilitation helps patients overcome “learned non-use”, where impairment of a disabled limb is exacerbated by disuse (Taub et al., 1993).

Following stroke, many patients learn to not use the impaired limb and instead

compensate by using the other “good” limb to carry out routine activities. Studies involving therapies that encourage or force use of the impaired limb (e.g. constraint-induced movement therapy, body-weight supported treadmill) have shown substantial improvement in function (Wolf et al., 1989; Dean and Shepherd, 1997; Mark and Taub, 2004; Peurala et al., 2005; Ng et al., 2008; Peurala et al., 2009). Finally, functional imaging studies have shown that rehabilitation has a direct impact on neuroplasticity, facilitating cortical reorganization in both the ipsilesional and contralesional hemispheres (for review see Hodics et al., 2006). Adjunct therapies that stimulate the cortex (e.g. transcranial magnetic stimulation) appear to augment the neuroplastic response to rehabilitation and enhance recovery (Khedr et al., 2005; Takeuchi et al., 2005; Kim et al., 2006; Takeuchi et al., 2008; Khedr et al., 2009).

Clinical studies have confirmed a number of key principles gleaned from animal models that appear to be essential in effective post-stroke rehabilitation. Firstly, clinical research has echoed the findings that initiating rehabilitation as early as possible is integral to optimizing recovery. Patients who receive therapy earliest tend to make better gains and achieve better outcomes (Horn et al., 2005; Maulden et al., 2005; Salter et al., 2006). Recent recommendations suggest beginning rehabilitation during hospitalization, even if not intense at first (Kelley and Borazanci, 2009). Secondly, intensity of rehabilitation correlates significantly with final outcome, and challenging the patient at all stages of recovery appears to push many beyond the recovery plateaus they might otherwise have reached (Kwakkel et al., 1997; Nelles, 2004; Teasell et al., 2009). Thirdly, task-specific

interventions not only lead to improved performance on most outcome measures but also tend to translate these improvements to better function in activities of daily living (Dean and Shepherd, 1997; Langhammer and Stanghelle, 2000; Hallett, 2001). Unfortunately, many current systems of stroke care fail to fully incorporate these principles and thus fail to provide optimal benefits to patients.

1.6.4 Rehabilitation as adjunct therapy

A recent line of research has suggested that in addition to the direct benefits of rehabilitation following stroke, it may also be useful as an adjunct therapy to enhance the effects of other treatments – especially cell-based therapies (for review see Johansson, 2000). For example, a combination of enriched environment and voluntary running increased the survival and migration of subventricular zone stem cells transplanted near cortical lesions in rats and improved functional recovery on the cylinder test of forelimb asymmetry up to 30 days post-ischemia (Hicks et al., 2007). Similar effects were not seen at more protracted time points (Hicks et al., 2008), indicating that transplant therapy and/or rehabilitation must be optimized in order to provide long-term benefit. Reaching therapy was also found to enhance the survival of fetal tissue grafts near cortical aspiration lesions and improve reaching performance in rats (Riolobos et al., 2001). Both enriched environment and reach training improve the functional recovery of rats with striatal lesions and cell grafts (Dobrossy and Dunnett, 2003, 2004, 2006).

1.7 Neurobehavioural Assessment

1.7.1 Tests of sensorimotor function

An array of neurobehavioural tests is available to assess functional outcome, impairment and recovery in rodents following ischemia. While some tests are used to measure overall sensorimotor function, others specifically assess forelimb function. Tests of skilled reaching ability are especially useful in studies using models of focal ischemia with damage to the forelimb motor cortex and/or dorsolateral striatum. While these tests generally require substantial training prior to injury, they are very sensitive to subtle forelimb impairments and can be used to monitor recovery over time. This is particularly important in rehabilitation studies, where the effect of treatment on skilled and unskilled motor functions may differ.

When assessing functional outcome and recovery following focal ischemia, it is important to use a battery of tests that is sensitive to a range of sensorimotor impairments. For lengthy experiments, such as rehabilitation studies, these tests must be reflective of impairments in a number of domains including overall sensorimotor function, forelimb movement and ability, and motor coordination. Additionally, these tests must be able to detect subtle changes in performance that are associated with functional recovery over a number of repeated test periods.

For experiments in this thesis, a test battery consisting of the Montoya staircase (skilled reaching), beam walking (motor coordination) and the cylinder test (forelimb asymmetry)

was used in Chapters 3 and 4. Together, these tests provide an accurate measure of both skilled and unskilled motor function and a combination of both general and forelimb-specific impairments. All three tests can be used repetitively to provide a sensitive measure of recovery over time. A neurological deficit score, which is sensitive to acute neurobehavioural impairment, was used in Chapter 2. This experiment utilized a relatively short treatment period, and impairment and recovery were best reflected using this measure. These and other tests of sensorimotor function are described in detail below.

1.7.1.1 Cylinder test

The cylinder test is a simple but effective measure of forelimb asymmetry (Schallert et al., 1997). Animals are placed in a clear, vertical cylinder and allowed to explore. While exploring, animals rear and support their body on the walls of the cylinder using their forepaws. Contacts with each forepaw are counted from a video recording. Normal rats use each forepaw approximately equally, but show a strong reliance on one paw (ipsilesional) following focal ischemia. Depending on the model of ischemia used, there may be some spontaneous recovery of symmetric use (i.e. shift back towards equal use) noted over time, however some level of impairment often persists and treatment-induced recovery has been reported (Biernaskie and Corbett, 2001; Hicks et al., 2007). No training or prior exposure to this test is required, making it especially practical. However, because animals are more motivated to explore in a novel environment, the decline in exploratory behaviour after repeated exposure to this test makes it less useful in lengthy

studies with numerous test periods. It is important to note that unlike many other tests of forelimb function, use of the impaired forelimb is neither forced nor motivated in this test and as such may show a unique recovery profile. Interestingly, it has been shown that the spontaneous nature of forelimb use in the cylinder test may be useful for unmasking persistent impairments that could otherwise be masked by compensatory mechanisms in other tests that force or motivate the animals to use the impaired limb (Clarke et al., 2005).

1.7.1.2 Foot fault assessment

Several variations of tests for foot fault are available, but in general animals are trained to cross a structure (beam, horizontal “ladder”) that requires balance, coordination and precise foot placement. Sessions are video recorded and errors (i.e. foot faults) are quantified. The most commonly used test involves having animals cross a raised beam (Kolb and Whishaw, 1983; Biernaskie and Corbett, 2001; Clarke et al., 2005) that may or may not have a ledge, and may remain a consistent width along its entire length or it may taper to become narrower at the far end. More difficult versions of the test use a round beam, and one version uses a rotating beam (Ohlsson and Johansson, 1995). A similar test uses a horizontal “ladder” instead of a beam, with the rungs spaced unevenly to provide more or less challenging crosses (Metz and Whishaw, 2002). While the precise scoring protocol for these tests varies between research teams, it is generally possible to assess forelimb and hindlimb separately or together (i.e. “total foot faults”).

Experiments in this thesis used a ledged, tapered beam in order to assess foot faults. We have previously shown that this test is sensitive to impairment following focal ischemia (Clarke et al., 2007b), and personal experience with several variations of the beam-traversing task indicates that this version has several advantages. Importantly, animals are more easily trained to cross a tapered beam and less likely to require “physical motivation” (i.e. nudging or forcing the animal to move forward) during the crossing. A reluctance to cross or frequent stopping due to fear is a common problem with some versions of this task, and is problematic for subsequent analysis. Secondly, the ledge on this beam allows animals to make relatively “major” foot faults without losing balance and/or falling off the beam. Again, this can be a problem in some versions of the task, making crossing difficult and aversive for severely impaired animals and leading to problematic analysis. Finally, the dark box positioned at the end of our tapered beam encourages most animals to make the crossing. Other appetitive cues (e.g. home cage) have also been used with similar success.

1.7.1.3 Rotarod test

Similar to the foot fault test, the rotarod is used to measure balance and walking ability (Rogers et al., 1997), and has been used routinely with both mice and rats. It involves placing an animal on a rotating cylinder that typically accelerates at a fixed rate over time. The time until the animal falls off the cylinder (if at all) is recorded. While application of this test is simple and requires little training, it is a very basic measure of motor function and provides little insight into the impairment being assessed. There

appears to be significant spontaneous recovery on this test using most models of ischemia, making it of limited value in studies of rehabilitation.

1.7.1.4 Neurological deficit score / forelimb placement

The neurological deficit score is a battery of several simple tasks to assess overall neurobehavioural function and impairment. Variations of this battery abound, but usually include tests such as limb retraction, beam walking ability, spontaneous circling, and ability to grasp/hang from an elevated bar with the forepaws (Zhang et al., 2000). Similarly, tests of forelimb placement are often used to assess acute sensorimotor and proprioception impairment following focal ischemia. This battery involves a series of very simple tasks that determine an animal's ability to respond to various stimuli (e.g. stimulation of the vibrissae, displacement of the forepaws) by moving the forelimb (De Ryck et al., 1989; Schallert et al., 2000).

Both tests are graded on a scale and summed to provide a relative measure of function. While the tests are quick and easy to perform, they are subjective and caution should be used in interpreting their relationship to more complex sensorimotor function. Additionally, impairments measured using neurological deficit scores and forelimb placement tests often show significant recovery early after stroke (Murphy and Corbett, 2009), possibly limiting their usefulness in the chronic phase. For studies of long-term recovery and rehabilitation, more sensitive and task-specific tests should be used.

1.7.1.5 Montoya staircase test

The Montoya staircase test is a sensitive measure of skilled forelimb function (Montoya et al., 1991). It requires an animal to reach from a central platform for food rewards (e.g. Noyes pellets) located on a set of descending steps on either side of the body. The apparatus is designed such that the animals can only reach pellets on the left staircase using the left forelimb, and those on the right staircase using the right forelimb. This allows for distinct measurement of reaching ability for each limb, which is especially important in studies using focal ischemia where one limb is impaired.

The staircase test is a challenging task that requires substantial training prior to stroke. Using specific performance criteria is important in order to ensure that animals are able to perform the reaching task with high proficiency before inducing ischemia. Post-surgical staircase performance is often used to balance treatment groups based on impairment prior to treatment. Reaching impairments after focal ischemia are very persistent (Biernaskie and Corbett, 2001) and repeated testing at various time points after injury provides a sensitive measure of recovery and/or the effect of various treatments (Biernaskie and Corbett, 2001; Biernaskie et al., 2004; Clarke et al., 2007a).

1.7.1.6 Single pellet reaching test

In this test of skilled reaching, animals are trained to reach through a narrow slot to retrieve a small food reward placed in a well outside the reaching apparatus. This requires proficient aim and reach-grasp ability, and therefore generally necessitates substantial

training at the beginning of an experiment. In some apparatus, the wall can be positioned to encourage the animal to use a particular forelimb (e.g. contralesional) to perform the reaching task. Assessment usually includes recording the number of attempts required to retrieve a particular pellet and/or the total number of pellets successfully retrieved in a session. A notable problem with this test, compared with the staircase reaching test, is that it takes considerably more time to apply and fewer animals can be tested at the same time. The increased training required has led to great variability in the proficiency level obtained in most studies, raising some questions about the value of its use.

The single pellet reaching task is sometimes used to qualitatively assess the kinematics of reaching, both before and after an injury (Whishaw et al., 1993). A return of “normal” movement strategies can reflect true motor recovery as opposed to compensation where new strategies are developed in order to improve performance (McKenna and Whishaw, 1999; Whishaw, 2000; Gharbawie et al., 2005; Knieling et al., 2009).

Tray reaching, a variant of this task allowing the animal to reach for a number of pellets at one time placed in a larger well (Ploughman et al., 2007a), is often used as a motor learning/rehabilitation activity and less often as a functional outcome measure.

1.7.1.7 Other reaching tests

A number of other tests of skilled reaching have been described but are less utilized in the literature. For example, in the pasta matrix reaching test animals reach through a slot in

order to reach a vertical arrangement of uncooked pasta. The pattern of broken and retrieved pasta can be used to determine not only reaching performance but also the range and extension of the forelimb (Ballermann et al., 2001). The time required for a rat to remove a sunflower seed shell and the number of pieces of shell created has also been described as a sensitive measure of dexterity and forelimb impairment following brain injury (Gonzalez and Kolb, 2003).

1.7.1.8 Adhesive tape removal test

In this test, small pieces of adhesive tape or labels are placed on both forepaws and the order and time it takes for the animal to remove each piece is recorded. This is generally regarded as a test of sensory function, and a longer latency for removal reflects sensory impairment (Schallert and Whishaw, 1984). It has been found that animals may ignore tape placed on the forepaw opposite a unilateral lesion, suggestive of the sensory neglect sometimes observed in human stroke patients.

1.7.2 Tests of cognitive function

An equally diverse set of tests is available to measure cognitive function and recovery in rodents. While a detailed discussion of these tests falls outside the scope of this thesis, it is important to note that many models of ischemia may produce subtle cognitive impairments that can be measured using tests of spatial navigation and memory (e.g. Morris water maze, radial arm maze, Barnes' maze), associative learning (e.g. fear

conditioning), anxiety (e.g. elevated plus maze) and/or complex discrimination (e.g. novel object recognition, attentional set shift). A growing number of studies are recognizing the importance of cognitive impairment in experimental stroke, and are using these assessments alone or in combination with tests of sensorimotor function.

1.8 Histological procedures

A number of histological and immunohistochemical procedures were used throughout this thesis in order to assess various outcome measures after ischemia and/or rehabilitative interventions. The following section provides a brief description and overview of the markers used.

1.8.1 Cresyl violet

Cresyl violet is a nissl stain routinely used to identify neurons and cell nuclei. To that end, cresyl violet was used throughout this thesis in order to identify, delineate and estimate the volumes of ischemic lesions.

1.8.2 FosB/ Δ FosB immunohistochemistry

FosB and its splice variant Δ FosB are transcription factors expressed in the brain by the immediate early gene *FosB*. Unlike other related transcription factors of the Fos-Jun family, FosB/ Δ FosB is expressed in a region-specific manner following chronic

stimulation, and is relatively stable allowing detection for long periods of time after induction (Nestler, 1999). Recently, FosB/ Δ FosB was implicated as a potential marker of neural activity in the cortex following experimental ischemia and rehabilitative activity (Allred & Jones, 2008).

In a novel approach, we used immunohistochemical staining of FosB/ Δ FosB to measure use-dependent activation of intact / perilesional tissue following ischemia and rehabilitation. As discussed in Chapters 2 and 5, we have shown that FosB/ Δ FosB provides a valuable tool that may demonstrate key mechanisms underlying neuroplasticity and recovery after focal ischemia.

1.8.3 Fluoro Jade C

Fluoro Jade is a fluorochrome derived from fluorescein, and is used to label degenerating neurons (Schmued et al, 1997). While Fluoro Jade has been used routinely as a measure of ongoing neuronal death, the mechanism of staining remains unclear and therefore the specific type(s) of neuronal death/degeneration cannot be confirmed. Fluoro Jade C, the newest version of the dye, was developed to improve staining contrast and therefore provide superior visualization of labeled cells.

Since Fluoro Jade has been shown to sometimes stain other cell types, such as astrocytes (Anderson et al, 2003) and microglia (Damjanac et al, 2007), it is important to note that analysis and quantification in this thesis only included cells that could be identified

structurally as neurons. As such, it is possible that we underestimated the actual amount of ongoing neuronal degeneration since some labeled neurons may have been discounted during analysis due to their structure having been rendered unidentifiable or being only partially included in the section. However, it can be assumed that such underestimation was consistent across animals and did not significantly affect comparisons between treatment groups.

1.8.4 ED-1

ED-1 (the rat homologue of human CD68) antibodies are used to stain rat macrophages (Damoiseaux et al, 1994). Since the expression of this antigen increases during phagocytic activity it is especially useful to label activated microglia, which are an important component of the inflammatory response after cerebral ischemia (Zheng and Yenari, 2004). Since activated microglia have been shown to be involved in both acute (Davies et al, 1998) and chronic (Langdon et al, 2008) inflammation, it provides a useful marker to measure inflammation at various time points following ischemic onset.

1.8.5 Golgi-Cox

The Golgi-Cox stain is a method of impregnating entire neurons with silver chromate, allowing for morphometric analysis using light microscopy and tracing techniques or software. The precise mechanism of staining, and especially its selectivity, is unclear

however it remains a powerful tool that is routinely used to examine changes in neural structure.

Using a modified method published by Gibb & Kolb (1998), we analyzed dendritic arborization and spine densities in the contralesional forelimb motor cortex of ischemic rats following rehabilitation. Using these techniques, our lab has previously observed increased complexity of pyramidal neurons in layer V of this region following shorter durations of treatment (Biernaskie & Corbett, 2001; Biernaskie et al, 2004).

1.9 Specific rationales for thesis experiments

1.9.1 What are the effects of early enriched rehabilitation on the post-ischemic brain?

An analysis of neuronal activation, delayed cell death, and inflammation.

Both human and rat studies show that most recovery occurs early after stroke, and further recovery in the chronic period is very limited. Similarly, both physiotherapy in humans and enriched rehabilitation in rats have been found to be more effective when initiated early after stroke. Together, these findings suggest that the ischemic brain is “primed” for recovery in the acute and sub-acute phase, but it is unclear how early rehabilitation engages the brain to promote functional recovery and the neuroplastic changes that have been observed at more protracted time points. The aim of this first experiment is to determine whether early exposure to enriched rehabilitation influences the activity of intact neural networks that are thought to be involved in functional reorganization.

Neuronal activation was measured using FosB/ Δ FosB immunohistochemistry - a transcription factor that is expressed following chronic activation, making it a sensitive measure of neuronal activity after rehabilitation. Alternatively, rehabilitation might provide benefit by attenuating delayed cell death and/or inflammation. These possibilities were examined by measuring the impact of early rehabilitation on neuronal death (Fluoro Jade C), lesion volume (cresyl violet), and activated microglia (ED-1). Adult rats were subjected to endothelin-1-induced focal ischemia and placed into either standard housing, enriched environment or a combination of enriched environment and daily reach therapy starting 7 days post-ischemia. Neurological deficits were assessed throughout the study, and histological analysis performed after either 2, 5 or 10 days of treatment.

1.9.2 Do rehabilitation "tune-up" sessions enhance long-term functional recovery? An analysis of regular returns to enriched rehabilitation and its impact on functional recovery and neuroplasticity.

For the vast majority of stroke survivors, rehabilitation (i.e. physiotherapy and occupational therapy) is the only treatment option available. Following an initial phase of rehabilitation many patients experience a recovery "plateau" and limited further improvement. Many patients are subsequently discharged and return periodically for brief periods of therapy ... often lasting just days. It is unclear what, if any, benefit this periodic return to therapy has for functional recovery, and if the type and intensity of therapy is optimal for maintaining or further enhancing functional gains. The aim of this second experiment was to determine if a return to enrichment/rehabilitation (i.e. "tune-

up"), can produce beneficial changes in brain plasticity and further improve functional recovery. Adult rats were subjected to endothelin-1-induced focal ischemia and began enriched rehabilitation 7 days later. Following 9 weeks of treatment, all rats were returned to standard housing for 5 weeks followed by an intensive 2 week period of additional therapy. This was followed by another 5 weeks of standard housing and one final session of therapy. Functional outcome and recovery was assessed throughout the study using the Montoya staircase, beam-traversing and cylinder test of forelimb asymmetry. The neuroplastic response to treatment was measured in the contralesional forelimb motor cortex using Golgi-Cox analysis at the end of the study (24 weeks post-ischemia).

1.9.3 What role does enriched environment play in functional recovery? An analysis of varied daily exposure to enriched environments and its influence on recovery of skilled and unskilled motor function.

While research has clearly shown that an environment that promotes motor, sensory and cognitive stimulation is an essential component of post-stroke rehabilitation and recovery, evidence has suggested that it has differential effects on recovery of skilled and unskilled motor function. The aim of this third study was to examine the role of enriched environment in recovery when combined with reach therapy (i.e. enriched rehabilitation). A second aim was to determine if shorter durations of daily exposure are sufficient to promote recovery since this might increase the likelihood of clinical adoption. Adult rats were subjected to endothelin-1-induced focal ischemia and placed in enriched

environment for either 3, 6 or 20 hours per day combined with reaching therapy starting 7 days post-ischemia. Functional outcome and recovery was assessed using the Montoya staircase, beam-traversing and cylinder test of forelimb asymmetry.

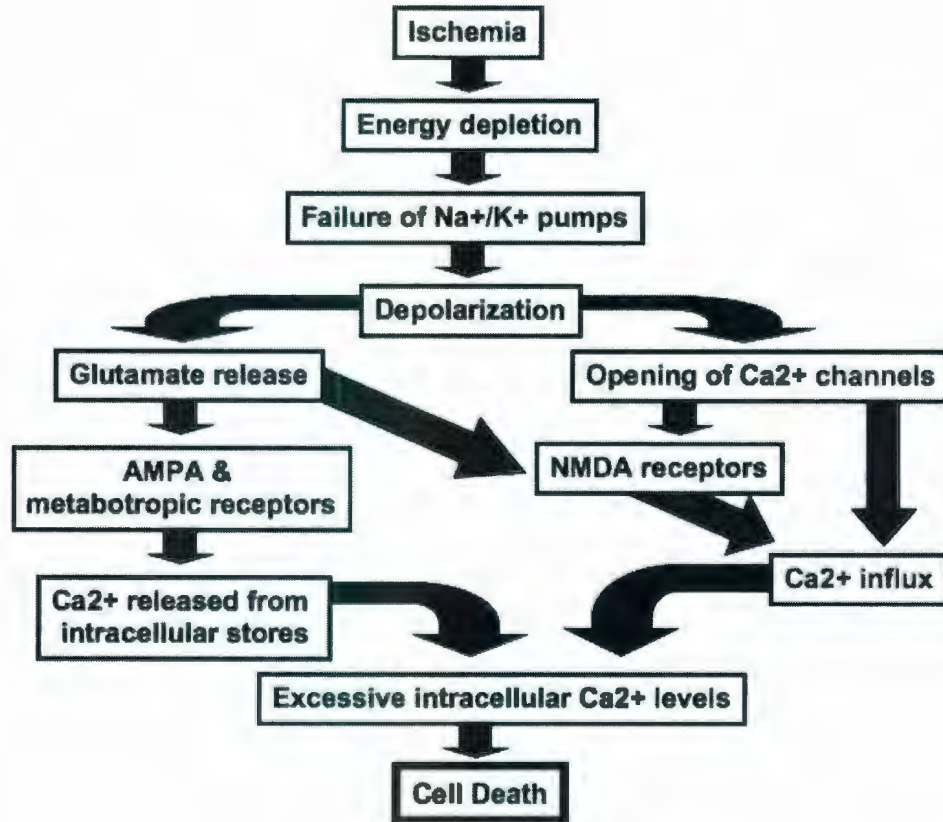


Figure 1.1 Summary of the ischemic cascade leading to cell death (adapted from (Lyden et al., 2002)).

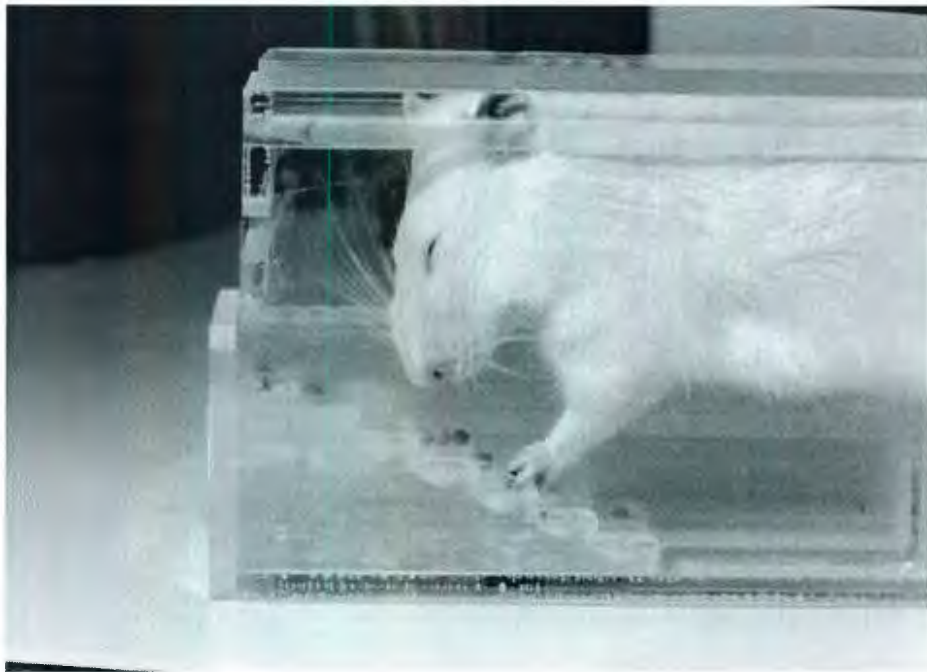


Figure 1.2 Montoya staircase test

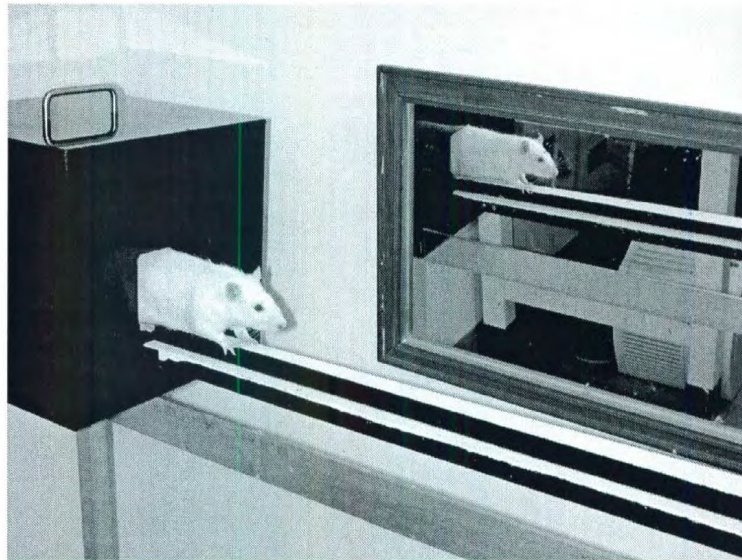


Figure 1.3 Beam-traversing test



Figure 1.4 Cylinder test of forelimb asymmetry

1.9 References

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Chapter 2 Mapping Functional Recovery Following Stroke Using FosB/ Δ FosB as a Marker of Use-Dependent Activation

2.1 Introduction

Most recovery occurs in the weeks immediately following stroke but many patients reach a plateau where further recovery is very limited (Duncan et al., 1992; Nakayama et al., 1994). Similarly, ischemic animals exposed to an enriched environment and daily reach training exhibit functional improvement that is most prominent in the early treatment period (Biernaskie and Corbett, 2001; Biernaskie et al., 2004) with little evidence of subsequent recovery (Clarke et al., 2009). A better understanding of the processes contributing to this early recovery is needed in order to optimize rehabilitation and extend the time window when significant functional benefit can be achieved.

Recovery after focal ischemia in rodents is associated with neuroplastic changes in perilesional cortex using traditional staining methods (Jones and Schallert, 1992; Brown et al., 2008) and advanced imaging (Zhang et al., 2005; Winship and Murphy, 2008, 2009). Reach training in monkeys (Nudo et al., 1996) and rats (Kleim et al., 2003) following motor cortex lesions leads to improved performance and increased motor map area relating to the impaired hand or forepaw. Rehabilitation also enhances functional recovery and promotes neuroplasticity in the intact cortex of ischemic rats (Jones et al., 1999; Biernaskie and Corbett, 2001). These findings indicate that task-specific therapy reorganizes uninjured cortex and promotes recovery.

Several lines of evidence clearly demonstrate that early rehabilitation is better and effectiveness decreases significantly with longer delays. Therapy initiated one month following focal ischemia in rats had little benefit compared to treatment started 2-3 weeks earlier (Biernaskie et al., 2004). Likewise, patients exhibit better functional recovery when admitted to rehabilitation programs early after stroke (Maulden et al., 2005; Salter et al., 2006).

While these findings suggest that events in the early phase following stroke are integral to the recovery process, the mechanisms underlying this critical time window are unknown. Ischemia leads to a transient upregulation of growth factors and genes involved in angiogenesis, neurogenesis and axonal sprouting, potentially “priming” the brain for repair processes (Carmichael et al., 2005). Nonetheless, these changes are relatively widespread and alone do not explain the enhanced recovery observed following treatment. Rehabilitation likely induces use-dependent activation of intact tissue that mediates neuroplastic changes and functional improvement. This possibility, while suggested by previous studies (Nudo et al., 1996; Dijkhuizen et al., 2001; Binkofski and Seitz, 2004), has not been directly tested in the earliest stages of post-stroke rehabilitation.

The current study used a novel approach to explore the effects of rehabilitation on neuronal activation following cerebral ischemia by measuring changes in FosB/ Δ FosB

expression. Unlike other markers of functional activity, FosB/ Δ FosB is activated by chronic or repeated stimulation and can be detected for days (McClung et al., 2004), making it sensitive to the effects of rehabilitation (Allred and Jones, 2008). Potential effects of rehabilitation on delayed cell death and inflammation were also investigated. Assessments were carried out following 2, 5 and 10 days of exposure to either enriched environment alone or combined with daily reach therapy. Functional impairment was assessed throughout the study using a neurological deficit score.

2.2 Materials and methods

2.2.1 Subjects

A total of 72 male Sprague-Dawley rats (Charles River Laboratories, Montreal QC, Canada) weighing 325-375g at the time of surgery were used. Animals were socially housed (2/cage) on a reverse 12h light/dark cycle, and all experiments were conducted in the dark phase. A total of 54 animals were exposed to endothelin-1 induced focal ischemia and allowed to recover in social housing, while 18 animals served as unoperated controls. Two ischemic animals died shortly after surgery, while one ischemic animal did not exhibit a neurological deficit and was excluded from the study. Seven days post-ischemia, the remaining animals were assigned to either enriched environment (EE; n = 17 ischemic, 6 intact), enriched environment and daily reach therapy (ER; n = 17 ischemic, 6 intact) or remained in standard housing (ST; n = 17 ischemic, 6 intact) for the

remainder of the experiment. Animals were sacrificed after either 2, 5 or 10 days of treatment. Final group numbers and survival times are summarized in Table 2.1.

2.2.2 Surgery

Animals were anesthetized with 3.5% isoflurane in 30% oxygen and 70% nitrous oxide, and maintained in a stereotaxic device with ~1.75% isoflurane. A midline incision was made in the scalp and 3 burr holes drilled at the coordinates (relative to bregma) given below. Focal ischemia was induced using injections of 400 pmol/ μ L endothelin-1 (CalBiochem, Hornby ON): 2 μ L at each of the forelimb cortical sites and 1 μ L at the striatal site (Windle et al., 2006).

- (1) Forelimb sensorimotor cortex – anteroposterior (AP) 0.0 mm/ mediolateral (ML) \pm 2.5 mm / dorsoventral (DV) -2.3 mm
- (2) Forelimb sensorimotor cortex – (AP) +2.3 mm / (ML) \pm 2.5 mm / (DV) -2.3 mm
- (3) Dorsolateral striatum – (AP) +0.7 mm / (ML) \pm 3.8 mm / (DV) -7.0 mm

2.2.3 Treatment Conditions

Standard housing consisted of a polycarbonate cage (48cm x 26cm x 20cm) with a section of PVC tubing (2 rats/cage).

Enriched environments (EE) consisted of large cages (90cm x 60cm x 60cm) equipped with an array of toys, tubes, ramps and ropes that provided sensorimotor stimulation (5-6

rats/cage). Environments were changed regularly to promote exploration. Food and water were available *ad libitum*. Reaching therapy during ER treatment involved providing access to a modified staircase reaching apparatus for 6 h/day (0900-1500) (Biernaskie and Corbett, 2001). Animals were removed from EE and placed in individual cages with free access to an apparatus baited with 14 g of pellets that can only be retrieved using the affected (i.e. contralesional) forepaw. The amount (in grams) of pellets retrieved was measured and replaced midway through and at the end of each session. Water, but no other food, was available during this period. (All animals were exposed to this reaching apparatus for three days prior to surgery in order to learn the task.)

2.2.4 Neurological Deficit Score (NDS)

A modified neurological deficit score (De Ryck et al., 1989) was used to assess gross sensorimotor impairment in animals before and 3, 6, 12 and 17 days after surgery. The test consists of five limb-placing tests which assess both fore- and hindlimb response to tactile and proprioceptive stimulation. In the first task, rats were placed on the edge of a table and forelimbs gently pulled down and released to check for replacement to the tabletop. The second task was the same but tested the hindlimbs. Both tests were repeated five times for each side of the body. The third task involved placing the forelimbs on the table surface and gently pushing the animal from behind. Control rats will resist the push equally with both forepaws, while ischemic animals will fail to do so with the impaired paw. This test was repeated twice, with each forepaw scored separately. The fourth task involved slowly lowering a rat held at the base of the tail toward the table surface.

Control rats reach to contact the table surface with both forepaws, while ischemic rats do so only with the less impaired forepaw and/or twist their body towards the side of the lesion. This test was repeated twice with each forepaw scored separately. The fifth and final task involved slowly moving the rat laterally toward the table edge and contacting it with the vibrissae. Control rats normally react by raising the ipsilateral forepaw to the table edge, while ischemic rats fail to do so. This evaluation was repeated three times for each side of the body. Tasks 1-4 were scored as follows: 2 points for normal response, 1 point for delayed and/or incomplete response, 0 points for no response; while task 5 was scored with 1 point for a response and 0 points for no response. The maximum score (i.e. no impairment) for each side of the body was 31. A subset of tasks (1, 3 and 5) was used to assess forelimb sensorimotor function, with a maximum score of 17.

2.2.5 Histology

After 2, 5 or 10 days of treatment, animals were deeply anaesthetized and transcardially perfused with cold 0.9% saline followed by 4.0% paraformaldehyde. Brains were removed, immersed in paraformaldehyde for 24 hours and subsequently stored in 20% sucrose in phosphate-buffered saline. Frozen sections (14 μm thick) were taken with a cryostat every 250 μm and slide-mounted for histological procedures. A series of sections spanning the lesion were stained with Cresyl violet, while consecutive series of sections were processed as described below.

For immunohistochemistry, sections were washed with phosphate-buffered saline, treated with 1.0% H₂O₂, blocked with 5.0% normal goat serum and incubated overnight at 4°C with either monoclonal mouse anti-rat CD68 (ED-1 for activated microglia; 1:1000; MCA341R, Serotec) or monoclonal rabbit anti-mouse FosB (102) (FosB/ΔFosB; 1:250; sc-48, Santa Cruz). The sections were then exposed to either goat anti-rabbit or anti-mouse biotinylated secondary antibodies (1:1000; Jackson Research Laboratories, West Grove PA, USA), incubated in 10 µg/mL extravidin (Sigma-Aldrich, Oakville ON, Canada) and reacted for 5 min in 3,3'-diaminobenzadine (Sigma-Aldrich). Primary omission controls were used to verify staining.

For Fluoro-Jade staining, sections were immersed in 1% NaOH/80% ethanol (5 min) followed by 70% ethanol (2 min), rinsed in distilled water and incubated in 0.06% KMnO₄ (10 min). Following a rinse in distilled water, sections were transferred to a 0.0001% solution of Fluoro-Jade C (30 min; Histo-Chem Inc, Jefferson AR, USA). Sections were rinsed in distilled water and allowed to dry on a slide warmer overnight before cover slipping in dim light.

Tissue loss (lesion and atrophy) was determined from Cresyl violet stained sections using ImageJ software, and calculated as follows:

Volume of tissue loss = volume of tissue remaining in the uninjured hemisphere – volume of tissue remaining in the injured hemisphere

Volume of a hemisphere = area of tissue remaining x distance between sections x number of sections analyzed

FosB/ Δ FosB positive nuclei and Fluoro-Jade positive neurons were counted throughout the perilesional area under a microscope using a 40x objective in combination with StereoInvestigator software (MicroBrightField Inc, Colchester VT, USA), while ED-1 positive cells were counted throughout the entire lesion area using a 20x objective. Four sections spanning the lesion were used for cortical analysis, while three sections were used for striatum. Three sections were also used to assess FosB/ Δ FosB immunostaining in the contralesional (i.e. uninjured) forelimb motor cortex. The area of interest was traced and the Fractionator used to randomly select ~20 sampling sites (100um x 100um) throughout the traced region and estimate a total number of positive cells or nuclei in that region (Langdon et al, 2010). The estimated population of positive cells or nuclei was used to calculate density (per mm²) for each section analyzed and averaged for each animal (a similar quantification of FosB/ Δ FosB positive nuclei was used in Kaufling et al, 2009).

2.2.6 Statistics

NDS data were analyzed using the Kruskal-Wallis test for non-parametric data, while histological data were analyzed using one-way ANOVA and Fisher's PLSD post-hoc analyses. Results were considered significant at $p < 0.05$. Intact animals were used to

determine control NDS scores and FosB/ Δ FosB expression in normal cortex (“contralesional” hemisphere was considered to be the hemisphere opposite the forelimb used for reaching in intact animals exposed to ER, and randomly assigned in those exposed to ST or EE). ANOVA revealed no differences among intact animals in either treatment, so these animals were combined for statistical analysis.

2.3 Results

2.3.1 Neurological Deficit Score (NDS)

Kruskal-Wallis analyses revealed no significant differences in functional recovery among treatment groups as assessed using either the total NDS score ($F_{2,14} = 1.53$, $p = 0.25$ after ten days treatment; maximum score = 31) or the subset of scores for forelimb function ($F_{2,14} = 0.98$, $p = 0.40$ after ten days treatment; maximum score = 17) at any survival time. All groups had similar impairments at three days post-ischemia, and all groups showed some improvement in gross neurological function over the course of treatment. Data for animals receiving ten days of treatment are shown in Figure 2.1.

2.3.2 Volume of Tissue Loss

ANOVA analyses revealed no significant differences in the volume of tissue loss among ischemic groups at either survival time (Table 2.2).

2.3.3 FosB/ Δ FosB Expression

ANOVA analysis revealed a significant difference among groups in FosB/ Δ FosB immunoreactivity in the perilesional cortex ($F_{2,14} = 4.40$, $p < 0.05$) after 10 days of treatment (Fig. 2.2). Animals exposed to ER treatment had significantly more FosB/ Δ FosB positive nuclei in the cortical regions surrounding the lesion than did either intact, EE or ST treated animals ($p < 0.05$). While similar trends were observed in both the perilesional striatum and contralesional forelimb motor cortex, neither difference reached statistical significance. No differences in FosB/ Δ FosB immunoreactivity were noted among treatment groups at earlier survival times.

ANOVA analysis also revealed that FosB/ Δ FosB expression in the contralesional hemisphere of all treatment groups was significantly lower than intact levels after both 2 ($F_{3,31} = 40.02$, $p < 0.0001$) and 5 ($F_{3,31} = 7.59$, $p < 0.001$) days of treatment.

Contralesional FosB/ Δ FosB expression in all groups was similar to intact levels after 10 days of treatment.

A qualitative analysis of FosB/ Δ FosB expression in the contralesional forelimb motor cortex showed that while positive nuclei were present in all cortical layers, they were more densely packed in the region corresponding to layer II/III following 10 days of ER treatment (Fig. 2.3). This layer-specific density was not evident in EE or ST groups at any time point. The stratification of cortical layers could not be reliably assessed in the damaged hemisphere.

2.3.4 Fluoro-Jade C

ANOVA analyses revealed no differences among treatment groups in Fluoro-Jade C staining (i.e. ongoing neuronal death) in perilesional tissues at any survival time (Fig. 2.4). Interestingly, a considerable amount of neuronal death remained ongoing after 10 days of treatment (i.e. 17 days post-ischemia) and did not show any reduction compared to earlier survival times.

2.3.5 ED-1 Expression

ANOVA analyses revealed no differences among treatment groups in the density of activated microglia (ED-1 positive cells) throughout the lesion area at any survival time (Fig. 2.5). Density of activated microglia remained relatively stable in the striatum across survival times, but showed a slight decline in cortex in all groups after 10 days of treatment (i.e. 17 days post-ischemia).

2.4 Discussion

We have shown for the first time that a combination of enriched environment and daily reach therapy (ER) increases the activity of perilesional tissue in rats following focal ischemia. The use of FosB/ Δ FosB immunohistochemistry to reflect this enhanced neuronal activity provides a novel means to demonstrate that task-specific therapy is an important component of early stroke therapy. These results parallel recent findings in

humans (Cramer et al., 1997; Cramer et al., 2000; Binkofski and Seitz, 2004), and suggest that recruiting intact brain tissue early after stroke may contribute to the neuroplastic changes and functional recovery observed after extended rehabilitation.

In accordance with earlier studies (Windle et al., 2006; Clarke et al., 2009), endothelin-1 applied to the forelimb motor cortex and dorsolateral striatum produced profound impairment in sensorimotor function. We previously demonstrated that 9 weeks of ER produced significant functional recovery following focal ischemia (Biernaskie and Corbett, 2001; Clarke et al., 2009). Despite the short treatment duration in this study, there was a trend to suggest that EE and ER treated animals were showing early signs of enhanced recovery compared to ST animals after just 10 days of rehabilitation. While earlier studies suggest that enriched environment must be combined with reaching therapy to achieve recovery in skilled reaching impairment (Biernaskie and Corbett, 2001), it is possible that the enriched environment alone is sufficient to lend some benefit to the more generalized sensorimotor function assessed by NDS. Use of a skilled reaching test might better detect functional differences among groups, although such differences may be more salient at later time points.

Importantly, 10 days of ER significantly increased FosB/ Δ FosB expression in perilesional cortex, while EE alone did not increase expression above levels observed in either ST or intact animals. Similar increases, though not reaching statistical significance, were observed in both perilesional striatum and contralesional forelimb cortex. These

findings suggest that task-specific rehabilitation targeting the main functional deficit (i.e. skilled reaching) increases the activity of cells around the lesion and in the intact hemisphere. This heightened activity at the cellular level is important in light of purely behavioural evidence from both animals (Biernaskie and Corbett, 2001; Kleim and Jones, 2008) and humans (Dean and Shepherd, 1997; Langhammer and Stanghelle, 2000) that task-specificity is an important component of effective rehabilitation.

The recruitment of intact cortex near the lesion during the acute phase of rehabilitation is likely a cardinal component of early recovery mechanisms. Functional imaging in ischemic rats suggests that increased activity in tissue surrounding the infarct is associated with improved behavioural outcome (Dijkhuizen et al., 2001). Reach training with the ipsilesional (i.e. unimpaired) forelimb reduced FosB/ Δ FosB expression in perilesional cortex and worsened behavioural outcome following focal ischemia (Allred and Jones, 2008). This further suggests that maintenance and/or enhancement of activity in remaining cortex is important to facilitate functional recovery.

The early activation observed here may precede neuroplastic changes and functional recovery observed in chronic, long-term experiments. Studies indicate that task-specific motor activity can alter motor maps in perilesional cortex. Training monkeys with small lesions of the primary motor cortex on a skilled reaching task led to preservation of intact cortical hand representation, and in some cases expansion into surrounding regions. These neuroplastic changes reflected improved reaching performance over the training

period (Nudo et al., 1996). Reorganization of perilesional cortex and enhanced recovery has also been observed in rats exposed to skilled reach training (Conner et al., 2005; Ramanathan et al., 2006). Similar expansions of motor maps have been detected in stroke patients treated with constraint-induced movement therapy, where use of the impaired arm and hand is encouraged by limiting use of the non-affected limb (Liepert et al., 1998; Liepert et al., 2000). Notably, these studies assessed cortical reorganization long after rehabilitation had commenced and thus it remains unclear whether behavioural recovery coincides temporally with the formation of new somatosensory maps. Interestingly, we previously showed that ischemic rats exposed to ER exhibit enhanced functional recovery beginning as early as 14 days after treatment onset (Biernaskie et al., 2004), corresponding with our current findings of increased neuronal activation in peri-infarct cortex after 10 days.

Cortical reorganization, increased dendritic complexity and synaptic plasticity also occur in the contralesional hemisphere of ischemic rats following rehabilitation, coinciding with functional recovery (Jones and Schallert, 1994; Biernaskie and Corbett, 2001). Recently, we showed that improvements in skilled reaching deficits achieved through rehabilitation were reversed by transiently inhibiting neural activity in the contralesional motor cortex (Biernaskie et al., 2005). These findings suggest that the intact hemisphere is intricately involved in functional recovery of the impaired forepaw despite having a minimal role in normal motor control of that limb. Results from neuroimaging studies in stroke patients support this theory, demonstrating increased recruitment of homotopic

motor regions in the contralesional hemisphere by movement of an impaired hand (Cramer et al., 1997; Cao et al., 1998). Disruption of the uninjured hemisphere using transcranial magnetic stimulation has also been found to impair movement of the paretic (ipsilateral) hand (Johansen-Berg et al., 2002).

The increase in FosB/ Δ FosB expression in the contralesional forelimb motor cortex occurred predominantly in layer II/III, which has been shown to be sensitive to manipulation in forelimb use (Adkins et al., 2002). Previous studies have shown that motor learning can increase synaptic density of both layers II/III (Kleim et al., 1996) and V (Jones, 1999). It is possible that more intense motor activity or longer treatment duration is required to increase FosB/ Δ FosB expression in layer V, or alternatively that other mechanisms are involved in the dendritic remodeling that has been observed in that layer following prolonged rehabilitation (Biernaskie and Corbett, 2001; Biernaskie et al., 2004). Regardless, our novel finding of enhanced neuronal activation in the contralesional cortex after 10 days of ER further supports the notion that the intact hemisphere is involved in recovery processes.

Increased FosB/ Δ FosB expression was not evident after either 2 or 5 days of treatment, implying that the activity is induced by repetitive therapy and not simply by a short period of motor activity. Further research should investigate how to optimize frequency, duration and intensity of rehabilitation, as they may have differential effects on neuronal recruitment and subsequent neuroplastic changes.

It is unclear if a similar activation would occur when rehabilitation is delayed. However we previously demonstrated that the effectiveness of ER decreases with time, and therapy started 30 days following stroke produces little or no functional recovery and no increased dendritic complexity as observed with earlier intervention (Biernaskie et al., 2004). Plasticity mechanisms are likely more active in the acute phase following stroke, and the recruitment of neural networks and facilitation of recovery by rehabilitation may be time sensitive (Murphy and Corbett, 2009).

Numerous Fluoro-Jade C stained neurons were present at 17 days post-ischemia (i.e. 10 days treatment) in all groups. This supports earlier findings that vulnerable cells surrounding the ischemic core continue to die for days after the initial injury (Hossmann, 1994; Back and Schuler, 2004), and suggests that the lesion may continue maturing significantly longer than previously thought. Indeed, although there were no significant differences among groups in the volume of tissue loss at any survival time, there was a trend to suggest that the infarct volume in EE and ER groups is larger after 10 days of treatment than at earlier time points. Previous studies have shown that ER does not affect lesion volume at more chronic time points (Biernaskie and Corbett, 2001; Biernaskie et al., 2004; Clarke et al., 2009). It is possible that the lesion matures more quickly in animals exposed to enriched environments despite obvious benefits for functional recovery and neuroplasticity in intact tissue (Risedal et al., 1999; Farrell et al., 2001).

There were no differences among groups in inflammation as measured by density of activated microglia. The inflammatory response to cerebral ischemia begins immediately after onset and peaks several days later (Morioka et al., 1993; Zhang et al., 1997; Stoll et al., 1998), suggesting that the optimal window for any effective anti-inflammatory effect might be earlier than the onset of our current treatment. Surprisingly, the density of activated microglia remained relatively constant up to at least 17 days post-ischemia. We recently showed that inflammation can be detected as long as 9 months after global ischemia (Langdon et al., 2008), and it is likely that inflammatory responses continue to play a role in the sequelae of cerebral ischemia weeks or months after stroke onset. It is possible that chronic microglial activation reflects protracted cell death, albeit relatively minor, or alternatively that microglia may be engaging in reparative processes (Crutcher et al., 2006).

Our results show that regions of intact peri-infarct cortex are activated after just a few days of ER, potentially reflecting the earliest phase of post-stroke recovery. This activation may lead to the longer-term neuroplastic changes and cortical reorganization that is hypothesized to mediate motor relearning and functional improvement. In the absence of effects on inflammation and delayed cell death following ischemia, enhanced neural activation may be one of the key mechanisms underlying the benefits provided by rehabilitation.

		ST	EE	ER
2 days treatment	Ischemic	6	5	6
5 days treatment	Ischemic	5	6	6
10 days treatment	Ischemic	6	6	5
	Intact	6	6	6

Table 2.1 Experimental groups

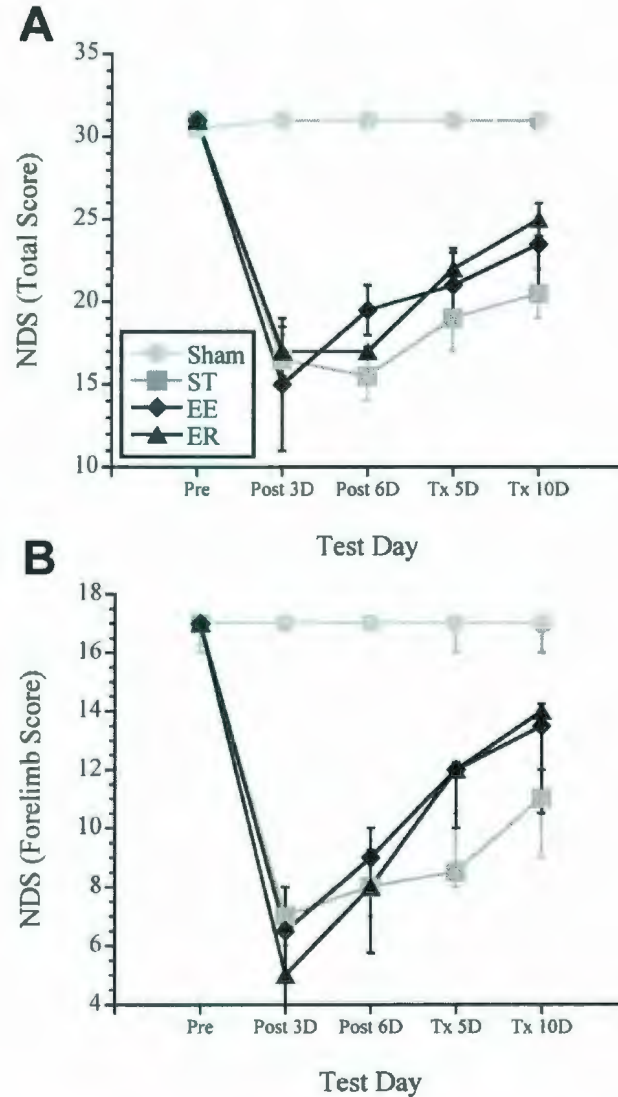


Figure 2.1 Neurological deficit score (NDS). All ischemic groups showed profound sensorimotor impairments post-surgery compared to intact animals on both total NDS (**A**) and on a subset of scores used to assess forelimb function (**B**). While all groups showed some recovery over time, there were no differences amongst groups. Data shown include only those animals receiving 10 days of treatment. Values are median scores with interquartile range.

Treatment		ST	EE	ER
2 days	Hemisphere	33.57 ± 8.04	30.33 ± 9.10	27.45 ± 6.43
	Cortex	28.47 ± 7.20	28.89 ± 7.74	22.74 ± 6.10
	Striatum	8.13 ± 0.97	7.44 ± 1.32	7.71 ± 1.23
5 days	Hemisphere	42.30 ± 9.90	32.40 ± 3.93	37.89 ± 10.17
	Cortex	29.64 ± 7.92	28.65 ± 3.03	32.19 ± 6.78
	Striatum	12.21 ± 1.05	7.77 ± 1.17	10.47 ± 1.62
10 days	Hemisphere	32.07 ± 4.92	60.78 ± 9.51	57.03 ± 13.00
	Cortex	22.08 ± 3.18	48.00 ± 6.27	40.80 ± 11.01
	Striatum	8.88 ± 1.05	10.86 ± 1.41	11.88 ± 2.04

Table 2.2 Volume of tissue lost (mm³). Data are mean ± SEM.

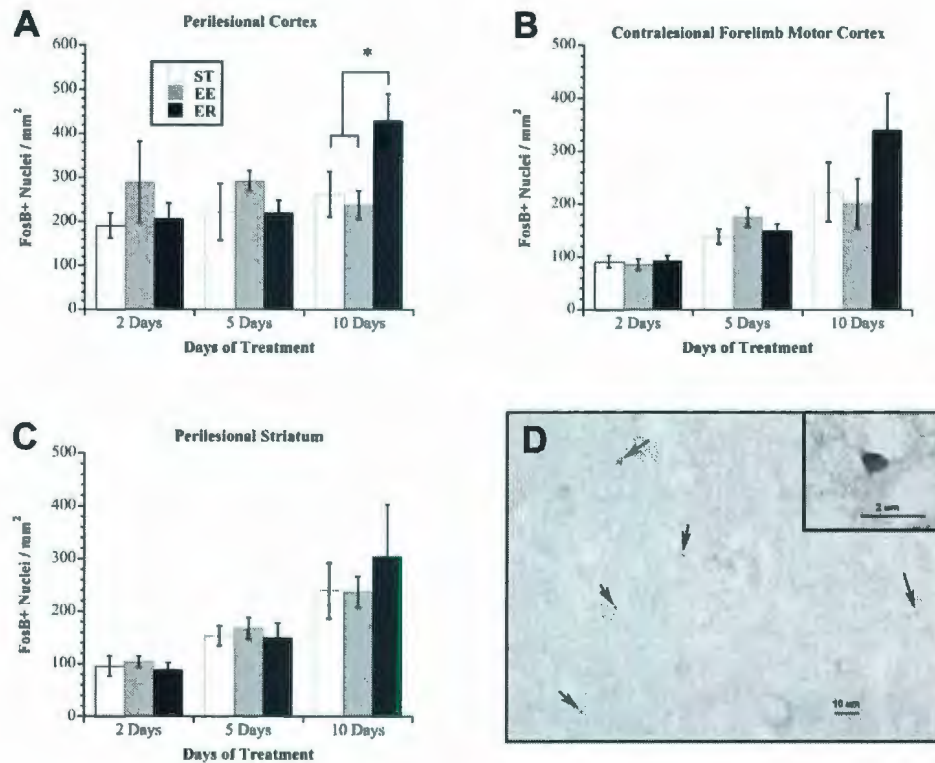


Figure 2.2 Use-dependent neuronal activation; FosB/ Δ FosB expression. (A) Animals exposed to ER treatment had significantly higher FosB/ Δ FosB expression in perilesional cortex than did those exposed to either EE or ST after 10 days of treatment. There were no differences amongst groups at earlier time points. Similar trends were observed in both perilesional striatum (B) and contralateral forelimb motor cortex (C), though not reaching statistical significance. (D) A representative photomicrograph (40x) illustrating FosB/ Δ FosB positive nuclei (black arrows) in perilesional cortex. Inset shows positive nucleus at 100x. Values are mean \pm SEM (* = $p < 0.05$).

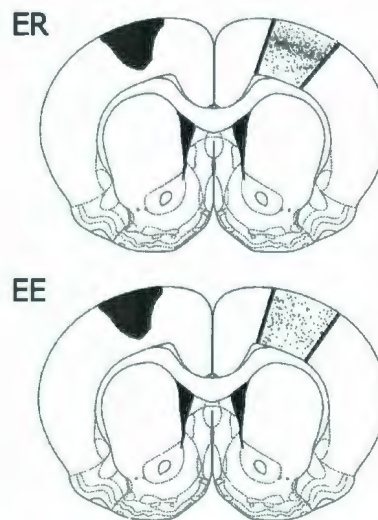


Figure 2.3 Representative diagrams showing FosB/ Δ FosB expression in the contralesional forelimb motor cortex of animals exposed to 10 days of treatment. Note the increased density of FosB/ Δ FosB positive nuclei (represented by black dots) in the region corresponding to cortical layer II/III of ER-treated animals. This increased density was not observed in EE or ST (not shown) groups.

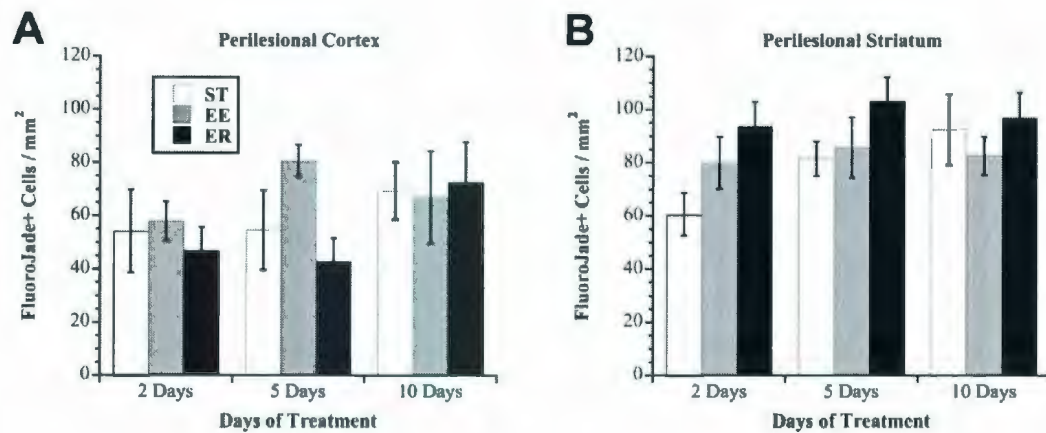


Figure 2.4 Delayed neuronal death; Fluoro Jade C. There was marked neuronal death occurring in both the cortex (**A**) and striatum (**B**) throughout the study period, up to 17 days post-ischemia (i.e. 10 days treatment). There were no differences amongst groups at any time point. Values are mean \pm SEM.

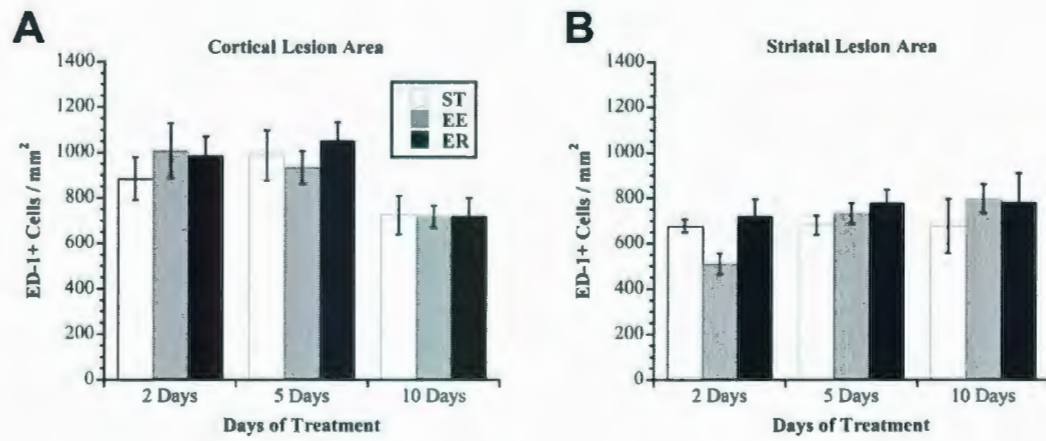


Figure 2.5 Inflammation; ED-1. There was marked ongoing inflammation (activated microglia) in and around the injured cortex (*A*) and striatum (*B*) throughout the study period. There were no differences amongst groups at any time point. Values are mean \pm SEM.

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Chapter 3 The effects of repeated rehabilitation “tune-ups” on functional recovery after focal ischemia in rats

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3.1 Introduction

Approximately 70-80% of stroke survivors are left with an upper-extremity impairment (Nakayama et al., 1994). Notably, 30% of these individuals remain severely disabled for the remainder of their lives (Muntner et al., 2002). For the vast majority of stroke patients, rehabilitation is the only treatment option available, since thrombolytic therapy is limited by its narrow time window and strict inclusion criteria (Barber et al., 2001).

Once patients appear to have reached a recovery “plateau”, rehabilitation is often discontinued and patients are discharged (Page et al., 2004a). A traditional view is that these “plateaus” represent maximal potential for recovery and that further therapy is fruitless (Jorgensen et al., 1995). However, when therapy includes progressively increasing intensity, duration and/or novel activities, some patients do show further improvement in motor function (Smith et al., 1999; Whitall et al., 2000; Page et al., 2004b). Thus these recovery plateaus may not represent maximal improvement, but instead an adaptation to the routine intensity and duration of traditional therapy – which may itself limit post-stroke recovery. Regardless, many stroke patients are discharged home instead of continuing to receive potentially beneficial therapy.

Many patients often return for periodic bouts of therapy, usually in an outpatient (Aziz et al., 2008; Lindsay et al., 2008) or home-based setting (Langhorne et al., 2005; Thorsen et al., 2005) lasting days or weeks. For example, some therapists employ constraint-induced movement therapy (CIMT), where patients are encouraged to perform routine and rehabilitative activities with the impaired arm while the other is restrained. This paradigm is used over a short period of time in a rotating manner of treatment/rest. Interestingly, CIMT has proved beneficial for chronic stroke patients months or years after reaching a recovery plateau (Dean and Shepherd, 1997; Page et al., 2001; Page et al., 2002; Rijntjes et al., 2008). Despite the regularity of this approach, it is unclear what if any benefit a periodic return to conventional therapy has for functional recovery, and if the status quo of routine physiotherapy is optimal.

The effect of rehabilitation has been well modeled in experimental stroke using a combination of enriched environment and rehabilitation (enriched rehabilitation; ER) (Biernaskie and Corbett, 2001). Briefly, following focal ischemia rats that are exposed to an enriched environment (a combination of social housing, complex environment and physical activity) as well as daily reach training show an improvement in sensorimotor performance compared to “untreated” rats. This recovery is accompanied by neuroplastic changes (e.g. increased dendritic branching) that are dependent on the timing of intervention (Biernaskie et al., 2004). Notably, this reflects only the early phase of clinical rehabilitation and animal models have yet to investigate the effects of the periodic return to therapy that some stroke patients experience. Similarly, it remains

uncertain if a secondary therapeutic intervention, such as a return to ER, can produce beneficial changes in brain plasticity that might further improve functional recovery.

In this study, the ER regimen described above was followed by rotating periods of non-treatment (i.e. standard housing) and brief returns to therapy (i.e. “tune-up” therapy). The “tune-up” (TU) therapy was more intense and enriching than ER because previous studies showed that ER alone has little or no benefit if initiated weeks after ischemia (Biernaskie et al., 2004). In order to incorporate progressive treatment intensity, animals were exposed to increasingly complex living environments and an increasingly challenging reaching task throughout TU periods. Functional outcome and recovery were assessed throughout the study using a series of well-established sensorimotor tests that measure skilled reaching (staircase test) and limb use (beam-traversing and cylinder tests).

3.2 Materials and methods

All procedures complied with regulations of the Canadian Council on Animal Care and were approved by the Institutional Animal Care Committee at Memorial University.

3.2.1 Subjects

A total of 62 male Sprague-Dawley rats (Charles River Laboratories, Montreal QC) weighing 325-350 g at time of surgery were used. Rats were socially housed (2/cage) on a reverse 12 h light/dark cycle and all experiments were done in the dark phase. Five rats

did not meet staircase training criteria and were eliminated. The remaining 57 rats were exposed to either endothelin-1 induced cerebral ischemia (n = 38) or sham surgery (n = 19). One sham-treated and 3 ischemic rats died due to surgical complications. The remaining 53 rats were either placed in ER (n = 18 ischemic, 9 sham) starting 1 week post-surgery or remained in standard housing and received no therapy (ST; n = 17 ischemic, 9 sham). Animals were balanced according to staircase reaching impairment assessed on days 5-6 post-ischemia and subsequently assigned to treatment groups. Following 9 weeks of this treatment, all rats were placed in standard housing for 5 weeks without therapy. Half the animals from each group were then given 2 weeks of intensive TU therapy, while the other half remained in standard housing. Another period of rest (5 weeks) and TU treatment (2 weeks) was administered to the same animals (*Fig. 3.1*). Neither treatment regimen affected the performance of sham animals, so shams were pooled for analyses. The resulting treatment groups were: SHAM (n=18); ST (n=8); ER (n=9); ST+TU (n=9); and ER+TU (n=9).

3.2.2 Surgery

Animals were anesthetized with 3.5% isoflurane in 30% oxygen and 70% nitrous oxide, and maintained in a stereotaxic device with ~1.75% isoflurane. A midline incision was made in the scalp and 3 burr holes drilled at the coordinates (relative to bregma) given below. Focal ischemia was induced in the hemisphere opposite the paw of best performance in the staircase test using injections of 400 pmol/ μ L endothelin-1

(CalBiochem, Hornby ON): 2 μ L at each of the forelimb cortical sites and 1 μ L at the striatal site (Windle et al., 2006).

(4) Forelimb sensorimotor cortex – anteroposterior (AP) 0.0 mm/ mediolateral (ML) \pm 2.5 mm / dorsoventral (DV) -2.3 mm

(5) Forelimb sensorimotor cortex – (AP) +2.3 mm / (ML) \pm 2.5 mm / (DV) -2.3 mm

(6) Dorsolateral striatum – (AP) +0.7 mm / (ML) \pm 3.8 mm / (DV) -7.0 mm

Sham surgeries consisted of anesthesia, incision, hole drilling and suturing in order to minimize mechanical damage.

3.2.3 Treatment Conditions

Standard housing consisted of a Plexiglas cage with a section of PVC tubing (2 rats/cage).

Enriched environments consisted of large cages equipped with an array of toys, tubes, ramps and ropes that provided sensorimotor stimulation (5-7 rats/cage). Environments were changed twice weekly to promote exploration. Food and water were available *ad libitum*. Reaching therapy during ER treatment involved providing access to a modified staircase reaching apparatus (Biernaskie and Corbett, 2001) for 6 h/day (0900-1500), 5 days per week. Animals were removed from enriched environments and placed in individual cages with free access to an apparatus baited with 14 g of pellets that can only be retrieved using the affected (i.e. contralesional) forepaw. The amount (in grams) of

pellets retrieved was measured and replaced midway through and at the end of each session. Water, but no other food, was available during this period.

The TU treatment consisted of 2 weeks of therapy providing enhanced enriched environments and structured sensorimotor/cognitive activities. Animals were exposed to one of four therapeutic environments and activities twice per day in a rotating fashion (e.g. enriched environment 0900-1300; running wheels 1300-1700). The enriched environments (4 h/day; 3 days/week) were more complex than those used during ER with a wider variety of toys and equipment, tactile surfaces to provide sensory stimulation, increased opportunity for climbing and other motor activity, and regular inclusion of aromatic substances (e.g. nutmeg, peanut butter) to provide olfactory stimulation. Environments were changed daily. Reaching therapy (3 h/day; 3 days/week) consisted of a tray reaching task in which rats could only retrieve pellets using their affected forelimb. Trays were successively raised to higher levels to provide an increasingly challenging task requiring better balance, and postural control (Ploughman et al., 2007a; Ploughman et al., 2009). Animals also received free access to running wheels attached to standard cages (4 h/day; 2 days/week), and to a variety of Hebb-Williams mazes (4 h/day; 2 days/week) in which a water bottle was hidden and treats (Fruit Loops®) were spread over the floor to encourage exploration and spatial navigation. Animals in standard housing were given similar amounts of pellets and Fruit Loops® on corresponding treatment days.

3.2.4 Staircase Reaching Test

Animals were mildly food-restricted (~90% of free-feeding body weight) throughout training and testing periods. Prior to surgery, animals were trained for 2 weeks (2x15 min trials/day, 5 days/week) to reach for 45 mg pellets (TestDiet, Richmond IN) in the staircase test, a sensitive measure of independent forelimb skilled reaching (Montoya et al., 1991). Reaching ability was measured by recording the number of pellets eaten, dropped and remaining on the steps of the staircase for each side at the end of each trial. Inclusion criteria required an average of ≥ 12 pellets eaten (out of a maximum 21) and a standard deviation of ≤ 2 on the last 8 training trials. Animals were re-tested at various time points throughout the study period (*Fig. 3.1*). Testing periods consisted of 2x15 min trials/day for 2 days.

3.2.5 Beam-traversing Test

Before surgery, rats were trained to cross an elevated tapered/ledged beam into a darkened box. Performance was videotaped and analyzed by calculating the slip ratio (number of slips/number of steps) of the forelimbs and hindlimbs separately. Steps onto the ledge were scored as a full slip (1.0) and a half slip (0.5) was scored if the limb touched the side of the beam (Schallert and Woodlee, 2005). The mean of 4 trials was used for statistical analyses. Animals were tested before (*Pre*) and at various time points post-surgery (*Fig. 3.1*).

3.2.6 Cylinder Test (*Asymmetrical Forelimb Use*)

Animals were placed in a clear Plexiglas cylinder (20 cm in diameter) situated on a glass tabletop and videotaped from below. Single (ipsilateral and contralateral) and bilateral forelimb wall contacts were recorded for a 5-minute period (or until a minimum of 15 wall contacts were observed). Contralateral forelimb use was expressed as: (contralateral forelimb contacts + $\frac{1}{2}$ bilateral forelimb contacts/total number of forelimb contacts) x 100 (Jones and Schallert, 1994; Biernaskie and Corbett, 2001). Animals were tested before (*Pre*) and at various time points post-surgery (*Fig. 3.1*).

3.2.7 Histology

At the end of behavioural follow-up (25 weeks post-surgery), animals were deeply anaesthetized and transcardially perfused with cold 0.9% saline. Brains were removed, stored in a modified Golgi-Cox solution for 14 days and then transferred to a 30% sucrose solution for 2-5 days. Brains were sectioned at 200 μ m on a vibratome, slide-mounted, and stained according to Gibb and Kolb (Gibb and Kolb, 1998).

For each animal, 6 apical and 6 basilar dendrites of layer V pyramidal neurons from the forelimb motor cortex of the contralesional hemisphere were traced using a 40x objective (effective magnification on computer monitor of $\sim 1700\times$) in combination with the NeuroLucida tracing system (MicroBrightField Inc., Colchester VT). A centrifugal branch order analysis (Coleman and Riesen, 1968; Biernaskie and Corbett, 2001) was carried out

using NeuroInvestigator software (MicroBrightField Inc.) to determine dendritic arbor complexity.

Dendritic spine densities were also examined in the same region. For each animal, 5 terminal basilar and 5 terminal apical branch segments were traced using a 100x objective (effective magnification of ~4250x) and spines indicated. Each branch segment was traced excluding approximately 10 μm at both proximal and distal ends. Spine density was calculated and expressed as number of spines per μm of dendritic length.

In order to be included for analysis, cell bodies and dendrites had to be located in the forelimb motor cortex region (approximately -0.80 mm to +1.50 mm AP from bregma), completely impregnated, unobstructed by other dendrites and/or blood vessels, and visible within the plane of the section.

As Golgi-Cox treatment does not lend itself well to volumetric analyses a 5-point damage score was used to assess infarct, as described previously (Ploughman et al., 2007b).

Briefly, a section with maximal injury was selected from each brain and a damage score assigned as follows: 0; no ischemic damage 1; 1–25% damage, 2; 26–50% damage, 3; 51–75% damage and 4; > 75% damage, compared to the intact cortex and striatum separately.

3.2.8 Statistics

Behavioural data were analyzed using repeated measures ANOVA, and a Dunnett's test was used to determine differences between groups. Damage scores were analyzed using the Kruskal-Wallis non-parametric test, and Golgi-Cox analyses using a one-way ANOVA. Results were considered significant at $p < 0.05$.

3.3 Results

Statistical analysis showed that all groups performed similarly before surgery, and that all ischemic groups were significantly impaired compared to sham animals at one week post-surgery on each of the behavioural tests. Sham animals were excluded from subsequent statistical analyses in order to examine treatment effects between ischemic groups only. Functional outcome was analyzed for the ER (ER vs ST; ending 10 weeks post-surgery) and TU (ER, ER + TU, ST and ST + TU groups; ending 25 weeks post-surgery) periods separately in order to determine the effects of both phases of treatment.

3.3.1 Staircase Reaching Test

Enriched rehabilitation. Reaching success was measured using the number of pellets retrieved and eaten on the side contralateral to the lesion, and expressed as a percentage of pre-surgery performance. A repeated measures ANOVA revealed a significant main effect of time ($F_{1,3} = 7.24$, $p < 0.005$) and a significant interaction of group \times time ($F_{1,66} = 4.19$, $p < 0.02$). Ischemic groups showed a profound impairment in reaching success at 1 week post-surgery (*Fig. 3.2A*; $p < 0.0001$ vs. shams). While reaching impairments

persisted in both groups throughout the treatment period, animals exposed to ER showed a steady improvement in reaching success such that they were significantly better than ST animals at the end of treatment ($p < 0.01$).

“Tune-up” therapy. A repeated measures ANOVA revealed a significant main effect of time ($F_{3,6} = 6.00$, $p < 0.0001$), but no effect of group or group x time interaction, indicating no effect of TU treatment (*Fig. 3.2B*).

3.3.2 Beam-traversing Test

Enriched rehabilitation. Performance was measured using the average number of foot faults per step while crossing the beam. A repeated measures ANOVA revealed significant main effects of group ($F_{1,33} = 5.45$, $p < 0.05$) and time ($F_{1,3} = 34.84$, $p < 0.0001$), and a significant interaction of group x time ($F_{1,66} = 3.37$, $p < 0.02$). Ischemic groups made significantly more foot faults per step than shams at 1 week post-surgery (*Fig. 3.3A*; $p < 0.0001$). However, ER animals improved during the treatment period, performing significantly better than ST animals at both the Mid ER ($p < 0.05$) and Post ER ($p < 0.0001$) test periods. No notable improvement was observed in ST animals.

“Tune-up” therapy. A repeated measures ANOVA revealed a significant main effect of time ($F_{3,6} = 16.60$, $p < 0.0001$), but no effect of group or group x time interaction, indicating no effect of TU treatment (*Fig. 3.3B*).

3.3.3 Cylinder Test

Enriched rehabilitation. Contralesional forelimb use was expressed as a percentage of total forelimb contacts on the wall of the cylinder during rearing and exploration. A repeated measures ANOVA revealed only a significant main effect of time ($F_{1,3} = 47.12$, $p < 0.0001$). Ischemic animals showed a strong reliance on the ipsilesional forelimb at 1 week post-surgery (*Fig. 3.4A*; $p < 0.0001$). Both ER and ST groups showed a similar improvement in contralesional forelimb use during the treatment period, and were not significantly different from sham animals by the end of treatment.

“Tune-up” therapy. A repeated measures ANOVA revealed a significant main effect of time ($F_{3,6} = 13.98$, $p < 0.0001$), but no effect of group or group x time interaction, indicating no effect of TU treatment (*Fig. 3.4B*).

3.3.4 Infarct analysis.

The area and extent of injury are described in Figure 3.5.

3.3.5 Spine Density

Spine density was measured on terminal dendrites in the contralesional forelimb motor cortex (*Fig. 3.6A*). A one-way ANOVA found no significant differences among groups in either apical ($F_{4,48} = 0.26$, $p = 0.91$) or basilar ($F_{4,48} = 0.14$, $p = 0.97$) dendritic spine density.

3.3.6 Dendritic Branching

A one-way ANOVA of branch order found no significant differences among groups in either apical ($F_{4,48} = 0.65$, $p = 0.63$) or basilar ($F_{4,48} = 0.64$, $p = 0.59$) dendritic complexity (*Fig. 3.6B*). However, there was a non-significant tendency for ST animals to have fewer basilar dendritic segments at each branch order, and ST animals were the only group to have no branch segments beyond the 7th order on basilar dendrites or beyond the 19th order in apical dendrites.

3.4 Discussion

Similar to previous studies (Biernaskie and Corbett, 2001; Biernaskie et al., 2004), we found that enriched environment combined with daily reach rehabilitation (ER) provided in the early post-stroke period improves motor function following focal ischemia in rats. Animals exposed to ER at 7 days after ischemia showed a steady improvement in motor function over the course of treatment, and performed significantly better than untreated (i.e. standard housed) animals on both staircase and beam-traversing tests at the end of 9 weeks of treatment. These results add to growing evidence that stroke patients should be provided with a stimulating environment in addition to intensive task-specific rehabilitation (Teasell et al., 2005). Many studies have shown that enriched environments encourage neuroplastic changes in the intact brain (Greenough et al., 1985; Kolb and Gibb, 1991), and promote recovery following various types of brain injury and stroke (Ohlsson and Johansson, 1995; Johansson, 1996; Johansson et al., 1997; Biernaskie and

Corbett, 2001; Komitova et al., 2005b). Exposure to complex living environments also raises levels of neurotrophic factors (Rowntree and Kolb, 1997; Dahlqvist et al., 1999), enhances dendritic branching (Biernaskie and Corbett, 2001; Johansson and Belichenko, 2002; Biernaskie et al., 2004) and increases neurogenesis (Komitova et al., 2002; Ehninger and Kempermann, 2003; Komitova et al., 2005a). Yet many stroke survivors, even in specialized treatment centers, spend much of their time inactive and alone with a lack of environmental stimulation (Keith and Cowell, 1987; Tinson, 1989; Lincoln et al., 1996; Bernhardt et al., 2004).

Importantly, our results confirm that ER is effective in a model of focal ischemia that specifically targets forelimb motor control, and which is known to be resistant to spontaneous recovery (Windle and Corbett, 2005). Interestingly, we saw a less striking improvement in reaching performance compared to our previous studies using the endothelin-1 middle cerebral artery occlusion (MCAO) model of ischemia (Biernaskie and Corbett, 2001), which often spares forelimb motor cortex and may be more amenable to rehabilitation-induced recovery. Standard housed animals (ST) showed no improvement in performance on either the staircase or beam-traversing tests, again confirming that this model produces persistent motor impairment with little spontaneous recovery. At the same time, 9 weeks of ER treatment produced significant recovery on both tests. It is crucial that models of stroke reflect elements of human stroke when studying rehabilitation and neural repair (Carmichael, 2005). Such models should produce injury patterns and persistent sensorimotor impairments common in human

stroke. Our model meets both of these criteria, and we posit that it is ideal to study not only the behavioural/functional sequelae of rehabilitation but also the neuronal correlates subserving recovery processes.

Despite the functional improvements observed, treated animals did not approach pre-surgical performance on the staircase test of skilled reaching suggesting that there is room for enhanced recovery and a need for further intervention. Therefore, it is important that this study investigated the effects of a “return to therapy”, where animals were exposed to additional periods of therapy in the chronic phase of post-stroke survival when sensorimotor recovery appeared to plateau.

It is unrealistic that stroke survivors will receive continuous structured therapy. Instead, most patients receive therapy in the sub-acute phase and are discharged home (where programs or self-directed therapy are often lacking). Some patients may receive subsequent periods of therapy, often in an outpatient setting, consisting of regular physical and/or occupational therapy provided over days or weeks (Aziz et al., 2008). While this “tune-up” approach is utilized in the clinical setting, it is not evidence-based. There are little or no data from clinical trials suggesting that these periodic returns to therapy provide any benefit to patients, and it has not been modeled experimentally.

We found no evidence that a “tune-up” approach was effective for motor recovery following focal ischemia in rats. Animals that received and benefited from early ER

therapy did not show any further improvement following TU therapy. It should also be noted that animals that received early ER but not TU did not show a decline in performance over the course of this study. One rationale for providing “tune-up” therapy to patients is to maintain gains in function that would otherwise deteriorate. However, both ER and ER+TU animals continued to perform at the improved levels observed following ER, and therefore maintained the recovery seen at that time regardless of subsequent treatment. However, it remains possible that with even more extended survival performance may have declined due to aging. Finally, no benefit of TU therapy was observed in animals that had received no previous treatment (ST+TU). This is not surprising, because as we have previously shown there is a crucial time-window for therapeutic intervention, and therapy initiated weeks following experimental ischemia has little or no functional benefit compared to treatment started early (Biernaskie et al., 2004). Additional interventions (e.g. stem cell therapies) aimed at increasing plasticity and/or extending this time-window may allow for more effective rehabilitation in the chronic phase.

No effect of treatment was observed on spine density in the contralesional forelimb motor cortex. This was not unexpected, as other studies have found no change in spine density after enriched environment and/or rehabilitation despite other neuroplastic effects (Biernaskie and Corbett, 2001; Kolb et al., 2003; Biernaskie et al., 2004; Monfils et al., 2005). *In vivo* imaging studies show that spines are very dynamic in response to stroke, changing in both number and morphology within minutes and hours of ischemic injury

(Brown et al., 2007; Brown and Murphy, 2008). Any changes in spine density or structure may be too transient for detection at the much later time points used in this study.

Notably, no significant effect of treatment was observed on dendritic branch complexity in the contralesional forelimb motor cortex. While other studies have found effects of ER on dendritic branching (Johansson and Belichenko, 2002) this has been detected immediately following weeks of continuous therapy. This study, however, examined branching after a more sporadic TU period and at a much later time point post-ischemia. Indeed, both human (Marshall et al., 2000; Butefisch et al., 2002; Feydy et al., 2002; Carey et al., 2005) and animal (Buchkremer-Ratzmann et al., 1996; Schiene et al., 1996) studies suggest that changes occurring in the contralesional hemisphere may be transient and more reflective of shorter-term compensatory responses after stroke.

Nonetheless, it is interesting that all treatment groups (ER and/or TU) *tended* to have a more complex basilar dendritic tree in the contralateral forelimb motor cortex than did the untreated (ST) group, indicating some degree of neuroplastic response to rehabilitation. It is possible that a more prolonged or intensive treatment paradigm might promote further dendritic branching, but further research is required to determine how this might be optimized in order to facilitate functional recovery. Additionally, functional recovery in the chronic phase after stroke may be more related to neuroplastic changes in

the peri-lesional cortex rather than the contralateral hemisphere. However, in the absence of such functional improvements, we did not investigate this possibility.

3.5 Implications

Our results clearly reaffirm the effectiveness of early therapeutic intervention for recovery of motor function following stroke. However, there appears to be little or no benefit of a return to therapy, as modeled here. At 6 months post-ischemia, there were no differences between treatment groups regardless of whether or not the animals received additional TU therapy.

Our findings imply that a periodic return to therapy may not be the most effective approach in post-stroke rehabilitation. It may be that patients would benefit more from an early intervention that is designed to progress in intensity more quickly, challenging the patient at each stage of improvement rather than allowing for recovery plateaus and subsequent discharge (Horn et al., 2005).

However, it is possible that our TU therapy itself was sub-optimal, and that a longer duration or greater emphasis on the primary impairment (i.e. reaching) would lead to further improvements. Furthermore, progressively more challenging and individually tailored clinical treatments are difficult to model in experimental environments. It is clear that more research on mechanisms of neuroplasticity and recovery in the chronic phase of

stroke is needed since many patients continue to make gains in function years after initial injury (Broeks et al., 1999; Hendricks et al., 2002; Kwakkel et al., 2002).

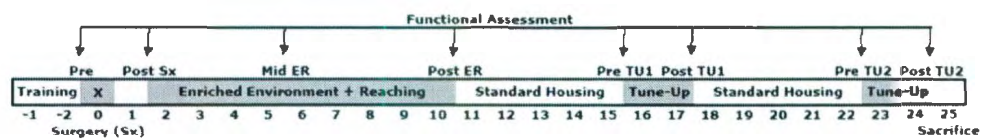


Figure 3.1 Experimental timeline. Time blocks are represented in weeks.

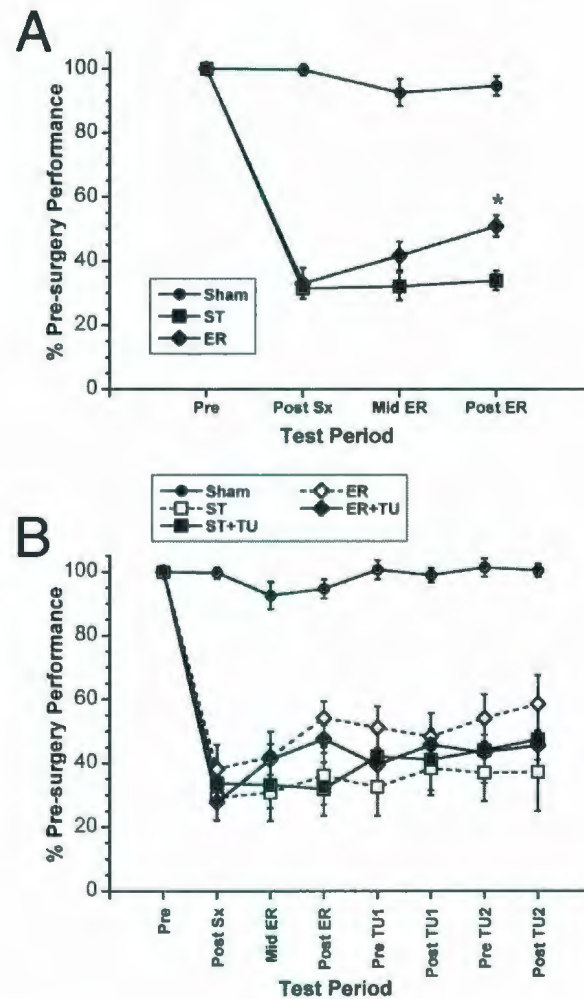


Figure 3.2 Staircase skilled reaching test. **(A)** Following ischemia (Post Sx), all animals were significantly impaired compared to sham animals. Animals in the ER group showed a steady recovery in reaching performance over the enrichment/rehab treatment period, and were significantly better than ST animals at the end of ER treatment (Post ER). ST animals showed no improvement during this period. **(B)** There were no significant differences among ischemic groups throughout the “tune-up” treatment period. Values are mean \pm SEM (* = $p < 0.01$; ER vs. ST).

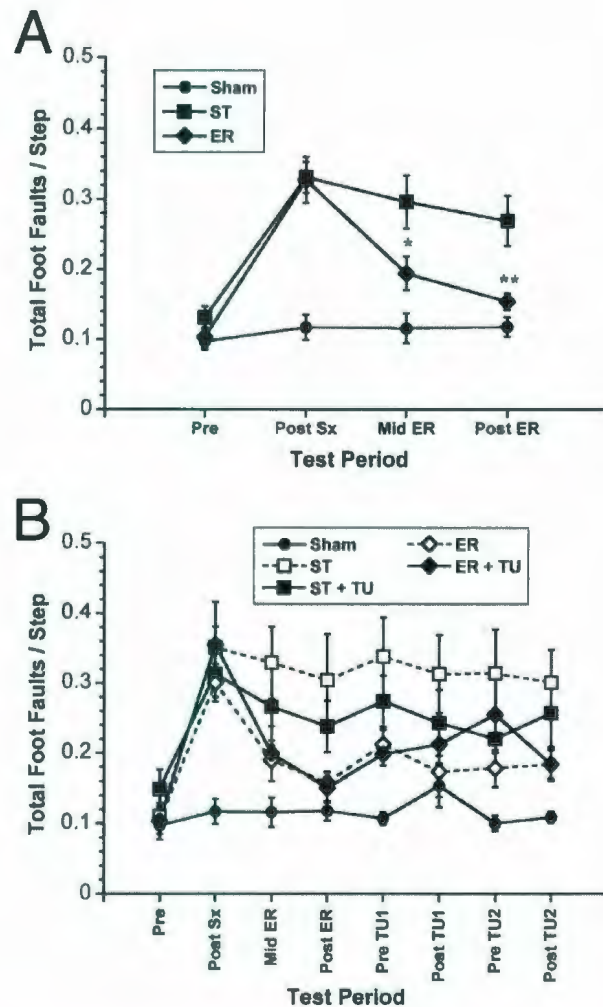


Figure 3.3 Beam-traversing test. **(A)** Both ER and ST animals made significantly more foot faults than sham animals following surgery (Post Sx). However, ER animals improved during the enrichment/rehabilitation treatment period such that they performed significantly better than ST animals and were not different than shams by the end of ER treatment (Post ER). **(B)** There were no significant differences among ischemic groups at any time point during the TU treatment period. Values are mean \pm SEM (* = $p < 0.05$, ** = $p < 0.0001$; ER vs. ST).

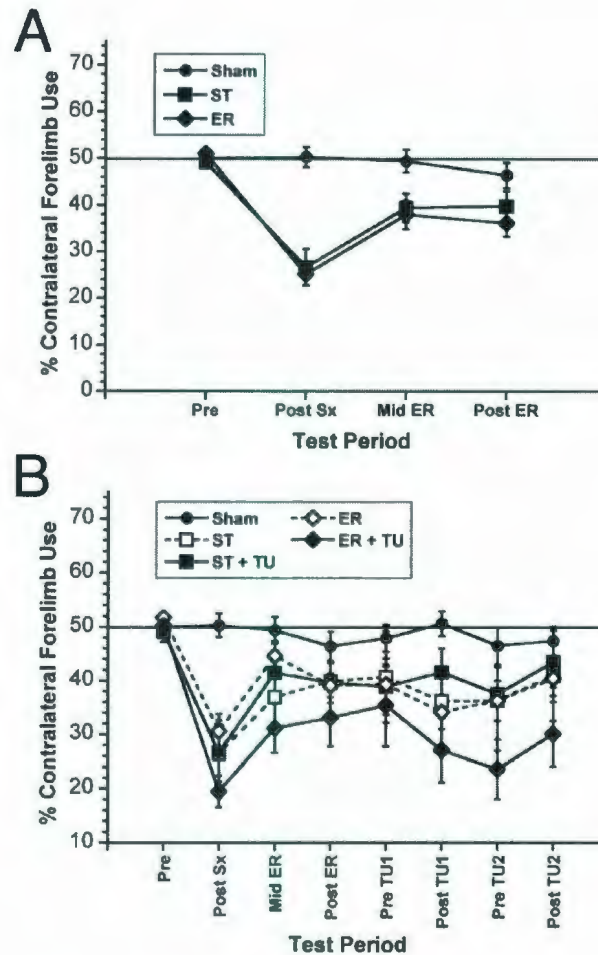


Figure 3.4 Cylinder test of forelimb asymmetry. **(A)** While all animals tended to use both forelimbs approximately equally prior to surgery (Pre), both ER and ST animals showed a significant reliance on the ipsilesional forelimb following surgery (Post Sx). Both groups showed a similar improvement in contralesional forelimb use over the ER treatment period, such that they were no longer different from shams by the end of treatment (Post ER). **(B)** There were no significant differences among ischemic groups throughout the “tune-up” treatment period. Values are mean \pm SEM.

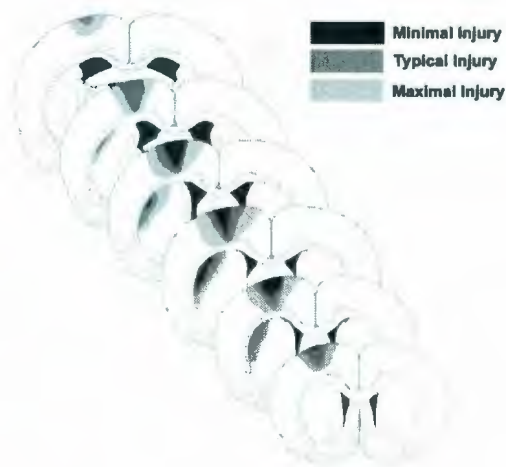


Figure 3.5 Representative illustration showing the area and extent of injury produced following endothelin-1 injection. The lesion typically affected the forelimb sensorimotor cortex and dorsolateral striatum. In animals with the largest lesions, incidental damage was observed in adjacent cortical regions. A 5-point damage score revealed no differences between treatment groups (Median score = 1.49; $H = 0.326$, $p = 0.955$).

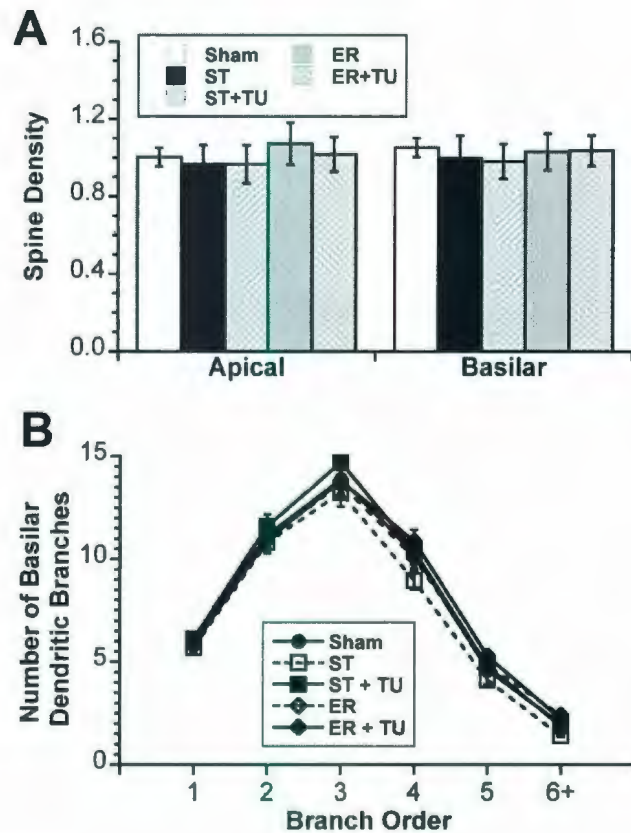


Figure 3.6 Golgi-Cox analysis. **(A)** There were no significant differences in apical or basilar dendritic spine density among groups at the end of behavioural follow-up (25 weeks post-surgery). **(B)** A dendritic branch order analysis found no significant differences in apical (*not shown*) or basilar dendritic branching among groups at the end of behavioural follow-up (25 weeks post-surgery). Values are mean \pm SEM.

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Chapter 4 The differential effects of enriched environment on recovery of skilled and unskilled motor function when paired with reaching therapy

4.1 Introduction

Enriched environments that combine social, cognitive and sensorimotor stimulation have been found to promote recovery of function in rodents after various types of brain injury (Hamm et al., 1996; Risedal et al., 2002; Steiner et al., 2006). Following focal ischemia enriched environments increase neurogenesis (Komitova et al., 2005), enhance neuroplastic remodeling (Johansson and Belichenko, 2002) and promote recovery of generalized sensorimotor function (Grabowski et al., 1993; Ohlsson and Johansson, 1995; Johansson, 1996). However, animal research (Biernaskie and Corbett, 2001; Maldonado et al., 2008) and clinical studies (Dean and Shepherd, 1997; Langhammer and Stanghelle, 2000; Bayona et al., 2005) have both indicated that task-specific therapy is required in order to produce significant recovery of skilled motor function, such as reaching with an impaired arm or forelimb. Indeed, when ischemic rats are exposed to a combination of enriched environment and daily reach training (i.e. enriched rehabilitation) they show marked recovery in both skilled and unskilled sensorimotor tasks (Biernaskie and Corbett, 2001; Biernaskie et al., 2004; Clarke et al., 2009).

The importance of a complex and stimulating environment has also been emphasized in clinical studies (Teasell et al., 2005; Roman, 2008). However, despite overwhelming evidence that enrichment is integral to effective rehabilitation, translation of these

principles into clinical practice has been slow. Recent research showed that stroke patients receiving inpatient treatment spent 60% of their day alone and inactive in a non-stimulating setting compared to only 12.8% engaged in rehabilitative activities (Bernhardt et al., 2004). Even patients on a dedicated stroke unit, touted as the gold standard in rehabilitative care, spent only 25% of their time taking part in interactive activities (Lincoln et al., 1996). It is clear that our current system of stroke care and rehabilitation has often failed to incorporate principles of enrichment and stimulation into the daily lives of patients. A better understanding of how enriched environments facilitate recovery will encourage incorporation of enrichment into rehabilitation programs.

A recent study demonstrated that rats exposed to middle cerebral artery occlusion and placed in enriched rehabilitation exhibited significantly better recovery on a test of skilled reaching compared to those that received either enriched environment or reaching therapy alone (Pawson et al., 2003). Since reach training alone has previously been shown to be sufficient to promote some recovery of reaching performance (Nudo et al., 1996; Gharbawie et al., 2005; Maldonado et al., 2008), the contribution of enriched environment to this enhanced recovery is unclear.

The goal of this study was to establish the role of enriched environment in functional recovery when combined with reaching therapy. Additionally, we aimed to determine if a shorter duration of daily exposure to enriched environment could produce similar functional recovery compared to 'standard' enriched rehabilitation. Starting one week

after focal ischemia, rats were placed in an enriched environment for 3, 6 or 20 hours per day and given access to reach therapy for 4 hours per day. Functional outcome and recovery were assessed before, mid-way and at the end of nine weeks of treatment using a battery of sensorimotor tests (staircase reaching, beam-walking and cylinder tasks). Infarct volumes were measured at the end of the study.

4.2 Materials and methods

All procedures complied with regulations of the Canadian Council on Animal Care and were approved by the Institutional Animal Care Committee at Memorial University.

4.2.1 Subjects

A total of 40 male Sprague-Dawley rats (Charles River Laboratories, Montreal QC) weighing 325-350 g at time of surgery were used. Rats were socially housed (2/cage) on a reverse 12 h light/dark cycle and all experiments were done in the dark phase. All rats were exposed to endothelin-1 induced focal ischemia. Five rats died due to surgical complications, and two were excluded due to lack of post-ischemic reaching impairment. The remaining 33 rats were balanced according to staircase reaching impairment assessed on days 5-6 post-ischemia and subsequently assigned to ER treatment groups starting seven days post-ischemia.

4.2.2 Surgery

Animals were anesthetized with 3.5% isoflurane in 30% oxygen and 70% nitrous oxide, and maintained in a stereotaxic device with ~1.75% isoflurane. A midline incision was made in the scalp and 3 burr holes drilled at the coordinates (relative to bregma) given below. Focal ischemia was induced in the hemisphere opposite the paw of best performance in the staircase test using injections of 400 pmol/ μ L endothelin-1 (CalBiochem, Hornby ON): 2 μ L at each of the forelimb cortical sites and 1 μ L at the striatal site (Windle et al., 2006).

- (7) Forelimb sensorimotor cortex – anteroposterior (AP) 0.0 mm/ mediolateral (ML) \pm 2.5 mm / dorsoventral (DV) -2.3 mm
- (8) Forelimb sensorimotor cortex – (AP) +2.3 mm / (ML) \pm 2.5 mm / (DV) -2.3 mm
- (9) Dorsolateral striatum – (AP) +0.7 mm / (ML) \pm 3.8 mm / (DV) -7.0 mm

4.2.3 Treatment Conditions

Enriched environments consisted of large cages equipped with an array of toys, tubes, ramps and ropes that provided sensorimotor stimulation (5-7 rats/cage). Environments were changed twice weekly to promote exploration. Food and water were available *ad libitum*. Reach therapy involved access to a modified staircase reaching apparatus for 4 h/day (0900-1300), 5 days per week. Animals were placed in individual cages with free access to an apparatus (Biernaskie and Corbett, 2001) baited with 14 g of pellets that can only be retrieved using the affected (i.e. contralesional) forepaw. The amount (in grams)

of pellets retrieved was measured and replaced midway through and at the end of each session. Water, but no other food, was available during this period.

At the end of each reaching session, animals were placed in enriched environments for either 3 (ER3; $n = 11$), 6 (ER6; $n = 11$) or 20 hours (ER20; $n = 11$). Animals in the ER20 group lived in enriched environment at all times (seven days/week), while those in the ER3 and ER6 groups were placed back in standard housing until the start of the next reaching session. Standard housing consisted of a polycarbonate cage with a section of PVC tubing (2 rats/cage). Treatment continued for nine weeks. Functional assessments were carried out before ischemia, before treatment, after four weeks of treatment, and at the end of treatment.

4.2.4 Staircase Reaching Test

Animals were mildly food-restricted (~90% of free-feeding body weight) throughout training and testing periods. Prior to surgery, animals were trained for 2 weeks (2x15 min trials/day, 5 days/week) to reach for 45 mg pellets (TestDiet, Richmond IN) in the staircase test, a sensitive measure of independent forelimb skilled reaching (Montoya et al., 1991). Reaching ability was measured by recording the number of pellets eaten, dropped and remaining on the steps of the staircase for each side at the end of each trial. Inclusion criteria required an average of ≥ 12 pellets eaten (out of a maximum 21) and a standard deviation of ≤ 2 on the last 8 training trials. Testing periods consisted of 2x15 min trials/day for 2 days.

4.2.5 Beam-traversing Test

Before surgery, rats were trained to cross an elevated tapered/ledged beam (1 m length, tapering from 10 cm to 2 cm) into a darkened box. Performance was videotaped and analyzed by calculating the slip ratio (number of slips/number of steps) of the forelimbs and hindlimbs separately. Steps onto the ledge were scored as a full slip (1.0) and a half slip (0.5) was scored if the limb touched the side of the beam (Schallert and Woodlee, 2005). The mean of 4 trials was used for statistical analyses.

4.2.6 Cylinder Test (Asymmetrical Forelimb Use)

Animals were placed in a clear Plexiglas cylinder (20 cm in diameter) situated on a glass tabletop and videotaped from below. Single (ipsilateral and contralateral) and bilateral forelimb wall contacts were recorded for a 5-minute period (or until a minimum of 15 wall contacts were observed). Contralateral forelimb use was expressed as: (contralateral forelimb contacts + $\frac{1}{2}$ bilateral forelimb contacts/total number of forelimb contacts) x 100 (Jones and Schallert, 1994; Biernaskie and Corbett, 2001).

4.2.7 Histology

At the end of the study, animals were deeply anaesthetized and transcardially perfused with cold 0.9% saline followed by 4.0% paraformaldehyde. Brains were removed, immersed in paraformaldehyde for 24 hours and subsequently stored in 20% sucrose in phosphate-buffered saline. Frozen sections (20 μ m thick) were taken with a cryostat

every 250 μm , slide-mounted and stained with Cresyl violet. Tissue loss (lesion and atrophy) was determined using ImageJ software, and calculated as follows:

Volume of tissue loss = volume of tissue remaining in the uninjured hemisphere – volume of tissue remaining in the injured hemisphere

Volume of a hemisphere = area of tissue remaining \times distance between sections \times number of sections analyzed

4.2.8 Statistics

Behavioural tests were analyzed using one-way and repeated measures ANOVA where appropriate. Histological results were analyzed using a one-way ANOVA. Results were considered significant at $p < 0.05$.

4.3 Results

4.3.1 Volume of Tissue Lost

The volume of tissue lost was calculated for the hemisphere, cortex and striatum (*Fig. 4.1*). ANOVA analysis revealed no significant differences among groups ($F_{2,30} = 0.195$, $p = 0.82$ for total hemisphere).

4.3.2 Rehabilitation Reaching Performance

Reaching performance during rehabilitation was expressed as the average amount of pellets retrieved per day during each week of treatment (*Fig. 4.2*). ANOVA analyses revealed no significant differences among groups during Week 4 (immediately prior to the Mid ER testing period; $F_{2,30} = 1.59$, $p = 0.22$). However, a significant difference was observed during Week 9 ($F_{2,30} = 3.48$, $p < 0.05$). Post-hoc analyses showed that animals in the ER20 group retrieved significantly more pellets than those in the ER6 ($p < 0.05$). The difference between ER20 and ER3 groups approached significance ($p = 0.062$).

4.3.3 Staircase Reaching Test

Reaching success was measured using the number of pellets retrieved and eaten on the side contralateral to the lesion, and expressed as a percentage of pre-surgery performance. All groups showed a profound impairment in reaching performance on days 5-6 post-ischemia (*Fig. 4.3A*). A repeated measures ANOVA revealed a significant main effect of time ($F_{2,2} = 4.56$, $p < 0.05$) indicating an improved performance over the treatment period. There were no significant differences among groups.

The percent recovery for each animal was calculated as the difference between performance at the end of the treatment period (End ER) and the post-ischemic impairment (Post Sx). While animals in the ER20 groups tended to exhibit better average recovery, an ANOVA revealed no significant differences among groups (*Fig. 4.3B*; $F_{2,30} = 0.74$, $p = 0.48$).

A Pearson Z analysis (transformed for normal data) revealed a correlation ($Z = 1.93$, $p = 0.05$) between reaching performance during the last week of treatment and the percent recovery in the staircase reaching test (*Fig. 4.3C*)

4.3.4 Beam-traversing Test

Performance was measured using the average number of foot faults per step while crossing the beam. All groups exhibited a profound impairment in performance on day 6 post-ischemia (*Fig. 4.4*). A repeated measures ANOVA revealed a significant main effect of time ($F_{2,2} = 44.35$, $p < 0.0001$), indicating an improved performance over the treatment period. There were no significant differences among groups, and all groups performed similar to baseline at the end of treatment.

4.3.5 Cylinder Test

Contralesional forelimb use was expressed as a percentage of total forelimb contacts on the wall of the cylinder during rearing and exploration. All groups exhibited a strong reliance on the ipsilesional (i.e. unaffected) forelimb on day 5 post-ischemia (*Fig. 4.5*). A repeated measures ANOVA revealed a main effect of time ($F_{2,2} = 7.32$, $p < 0.005$), indicating an increased use of the contralesional forelimb over the treatment period. There were no significant differences among groups, and all groups continued to exhibit a reliance on the ipsilesional forepaw at the end of treatment.

4.4 Discussion

The duration of daily exposure to enriched environment had no direct effect on the extent of recovery observed in any of the behavioural tests used in this study. All groups, regardless of treatment, showed similar recovery on the staircase, beam-traversing and cylinder tests. There was a trend to suggest that animals receiving more exposure to enriched environment (i.e. ER20) had slightly greater recovery in reaching success compared to baseline impairment, indicating that enriched environment may have augmented the rehabilitative effect of reaching therapy.

All groups showed moderate recovery of reaching function in the staircase test. This is not unexpected, as the access to daily reaching therapy did not differ between treatment groups despite the varying exposure to enriched environment. Previous research has indicated that while a combination of enriched environment and daily reach training enhances recovery of skilled reaching impairments (Biernaskie and Corbett, 2001; Clarke et al., 2009), enriched environment alone does not (Grabowski et al., 1993; Pawson et al., 2003). These findings suggest that task-specific therapy is required in order to promote functional recovery of skilled movement. Indeed, recent studies have demonstrated that skilled reaching, but not more generalized motor activity, is able to improve reaching performance and promote cortical reorganization in rats with unilateral motor cortical lesions (Kleim and Jones, 2008; Maldonado et al., 2008). Similar neuroplastic effects of

task-specific training, but not motor activity, have been observed in intact animals (Kleim et al., 1998; Remple et al., 2001; Kleim et al., 2002).

There was a non-significant trend suggesting that longer enriched environment exposure resulted in greater recovery from baseline reaching impairment in the staircase test. This suggests that enriched environment may augment the rehabilitative effect of reaching therapy. Indeed, further analysis showed that animals in the ER20 group retrieved more pellets in the reaching apparatus during the last week of rehabilitation than animals exposed to the same environments for less time. The precise nature of the relationship between enriched environment and performance in reaching rehabilitation is unclear, but may have to do with either enhanced motivation during the reaching session or, more likely, a generalization of motor skills gained/recovered during enriched environment exposure that improve reaching in the apparatus. Regardless, these results support the findings of a recent study demonstrating that a combination of enriched environment and daily reach therapy provides better recovery than either treatment alone (Pawson et al., 2003).

It is unclear why the improved reaching performance during the last week of rehabilitation did not translate into a significantly better performance in the staircase test at the end of treatment. However, there is a correlation between the amount of reaching success during this last week of treatment and recovery in the staircase test, indicating a direct relationship between performance in both reaching tasks. It is possible that the

reaching apparatus used for rehabilitation, due to the larger number of pellets available and the longer duration of reaching access, may be more sensitive to subtle improvements in reaching ability than the Montoya staircase. Alternatively, the effect in the staircase may have been just starting to emerge and longer treatment might have produced a significant difference between groups.

Other adjunct therapies have been found to enhance recovery in combination with rehabilitation. Cortical electrical stimulation in animals (Adkins-Muir and Jones, 2003; Kleim et al., 2003; Plautz et al., 2003; Teskey et al., 2003) and transcranial magnetic stimulation in humans (Khedr et al., 2005; Takeuchi et al., 2005; Kim et al., 2006) improve functional outcome after stroke when paired with task-specific therapies, indicating that increased cortical activity may facilitate motor learning and recovery. However, the persistence of these improvements has yet to be shown. Similarly, amphetamines administered prior to or concomitant with rehabilitative activities appear to provide some functional benefit in rodents (Feeney et al., 1982; Goldstein and Davis, 1990), although results from clinical studies have been equivocal (Martinsson et al., 2007; Goldstein, 2009). The incorporation of environments that stimulate patients physically, cognitively and socially into therapeutic programs and rehabilitation centres provides an effective and non-invasive means to enhance recovery. The potential cognitive and emotional impacts of such environmental exposure for patients, while difficult to assess in the experimental setting, are likely to prove equally beneficial. Depression (Dafer et al., 2008) and subtle cognitive impairment (Zhu et al., 1998; Grau-

Olivares and Arboix, 2009) are common amongst stroke survivors but are often not adequately addressed.

It is important to note that enriched rehabilitation had differential effects on outcome and recovery depending on the behavioural assessment used. All groups showed complete recovery on the beam-traversing task over the treatment period, despite profound impairment at one week post-ischemia. Nevertheless, very little recovery was observed on the cylinder test indicating that animals in all groups still exhibited a strong reliance on the ipsilesional forelimb at the end of enriched rehabilitation, and hence remained notably impaired.

The discrepancy between results on these two tests may be explained by either of two possibilities, or an interaction of both. Firstly, while both tests are used to measure innate, unskilled motor function they also differ importantly in that the beam task forces the rat to use its impaired limb(s) while the cylinder task does not. It is therefore possible that by the end of treatment the animals had recovered significantly in the “ability” to use the impaired limb(s) for tasks such as locomotion, and therefore performed better when forced to cross the beam. However, the lasting impairment may have presented itself as non-use during the cylinder test, when animals had the option of performing the task (i.e. exploration) without using the affected forelimb. This persisting over-reliance on the ipsilesional paw may be analogous to the phenomenon of “learned non-use” commonly observed in stroke patients (Taub et al., 2006).

Secondly, it is possible that the animals have developed compensatory movements that improve overall performance in walking without having truly recovered the motor function that was lost as a result of focal ischemia. Both animal research (Whishaw et al., 1991; Friel and Nudo, 1998; Metz et al., 2005) and clinical studies (Cirstea and Levin, 2000; Kwakkel and Wagenaar, 2002; Roby-Brami et al., 2003) suggest that compensation accounts for most of the functional improvement observed following stroke. In rodents exposed to focal ischemia, development of alternative movement strategies has been implicated in the spontaneous recovery of not only walking ability, but also forelimb use and skilled reaching performance (Metz et al., 2005). More recently, enriched environment was shown to promote compensatory strategies in a test of single pellet reaching but did not enhance recovery of normal motor movements (Knieling et al., 2009). Similarly, it has been suggested that physiotherapy approaches may allow and even encourage the development of compensatory movements in stroke patients in order to achieve functional improvement (Trombly and Wu, 1999). The use of compensatory mechanisms to improve walking ability would explain the improved performance on the beam-traversing task observed here, despite persisting impairments exhibited in the cylinder task.

Regardless of the mechanisms underlying the improved performance on the beam-traversing task, it is important to note that all treatment groups showed similar recovery. We have previously shown that untreated rats exposed to this model of focal ischemia

exhibit persistent impairments in the beam-traversing task (Clarke et al., 2009), indicating that the improvements observed here are due to rehabilitation and not spontaneous recovery. Together, these findings suggest that even short daily exposures to an enriched environment are sufficient to promote recovery of the more generalized sensorimotor functions involved in walking and foot placement compared to the skilled movements required in most reaching tasks.

The differential effects of treatment on these measures of sensorimotor function highlight the importance of using a battery of tests to assess outcome and recovery. It is essential to include tests that measure more than one domain of function, as recovery may be observed in some areas but not others (Kleim et al., 2007). It is also pertinent to include tests that measure outcomes directly related to both the impairment and the treatment – in this case, skilled reaching. Incorporation of a qualitative assessment may be used to determine whether improvements in performance are due to true motor recovery or compensatory strategies (Metz et al., 2005; Gharbawie and Whishaw, 2006).

Our results suggest that an enriched environment has distinct influences on the recovery of skilled and unskilled sensorimotor function after focal ischemia. While even short durations of environmental stimulation facilitate improvements in general motor tasks such as beam crossing, more extensive periods appear to enhance the rehabilitation of skilled motor impairments when paired with reaching therapy. The additive effect of environmental stimulation and reach training appears to be dose-dependant, with longer

daily exposure to enrichment promoting better reaching performance. This synergy of enrichment and task-specific therapy possibly explains the effectiveness of enriched rehabilitation in promoting functional recovery after brain injury. With this in mind, it is increasingly clear that post-stroke rehabilitation programs must provide stimulating environments that engage patients both during and outside of traditional therapeutic activities.

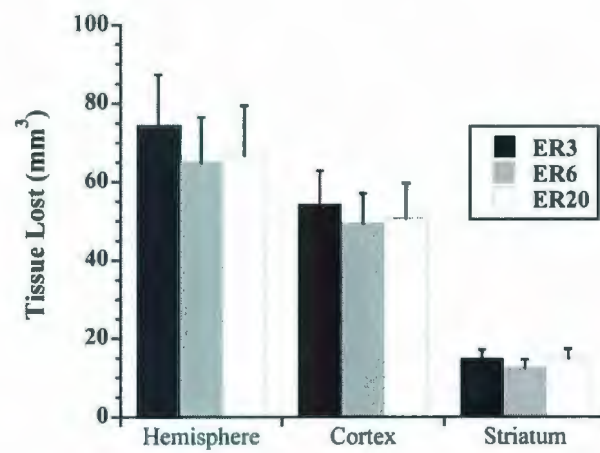


Figure 4.1 Infarct volumes. There were no differences among groups in the volume of tissue lost.

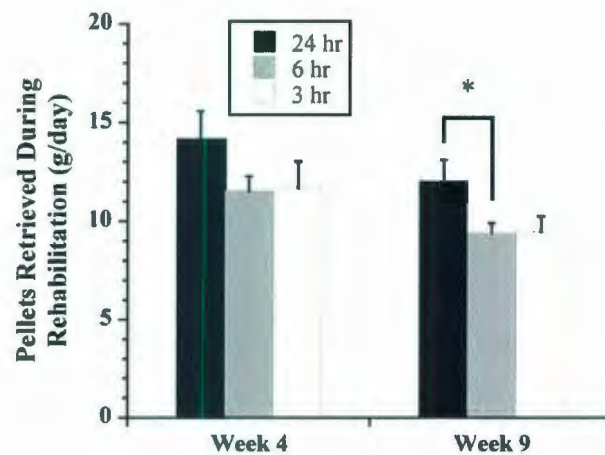
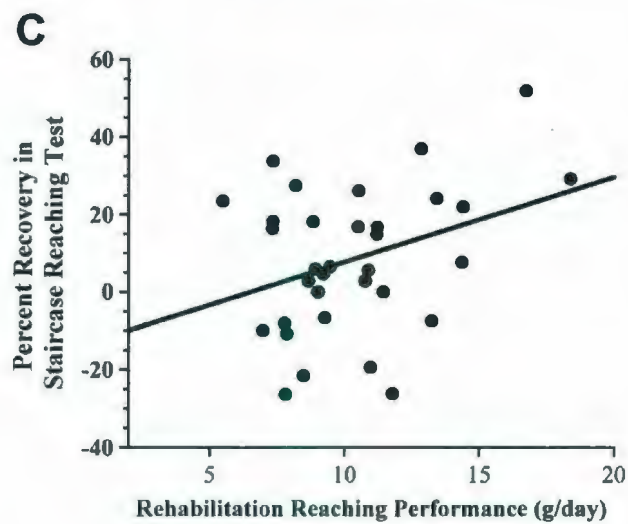
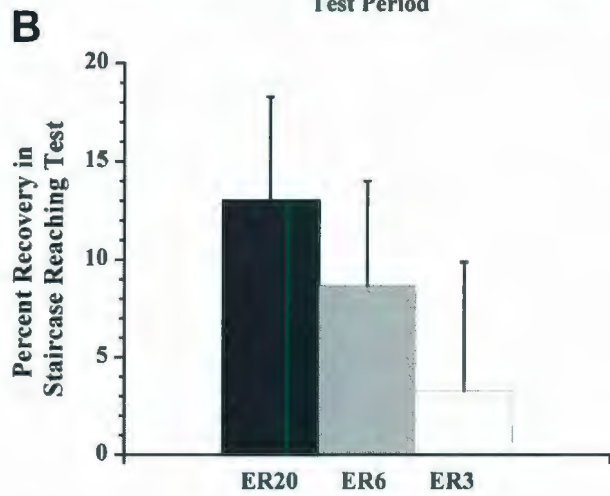
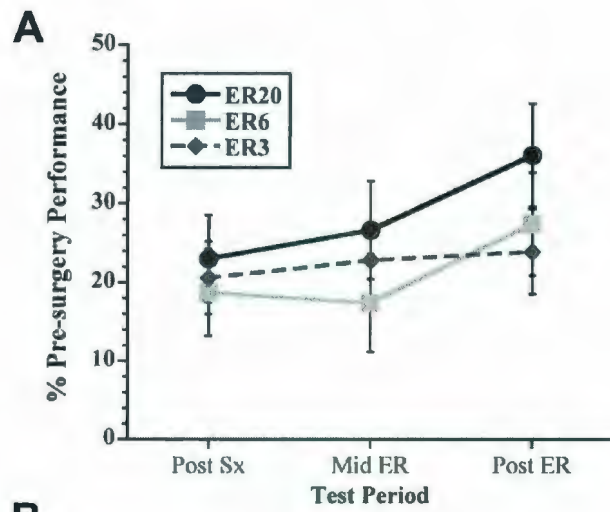


Figure 4.2 Reaching performance during rehabilitation. Animals in the ER20 group retrieved significantly more pellets during the last week of enriched rehabilitation (Week 9) than did those in the ER6 group ($p < 0.05$). The difference between ER20 and ER3 was similar and approached statistical significance ($p = 0.06$). A comparable trend was observed earlier in treatment (Week 4), but groups were not significantly different.

Figure 4.3 Staircase skilled reaching test. **(A)** All groups exhibited significant impairment of reaching ability on days 5-6 post-ischemia (Post Sx) and showed similar improvements in reaching performance throughout the treatment period. **(B)** Animals in the ER20 group tended (non-significantly) to exhibit slightly better recovery in the staircase reaching test than those in ER6 or ER3 groups at the end of treatment. This trend suggests that longer durations of daily exposure to enriched environment may be associated with better functional outcome. **(C)** There was a correlation ($p = 0.05$) between reaching performance during the last week of enriched rehabilitation (*see Fig. 2*) and recovery on the staircase reaching test.



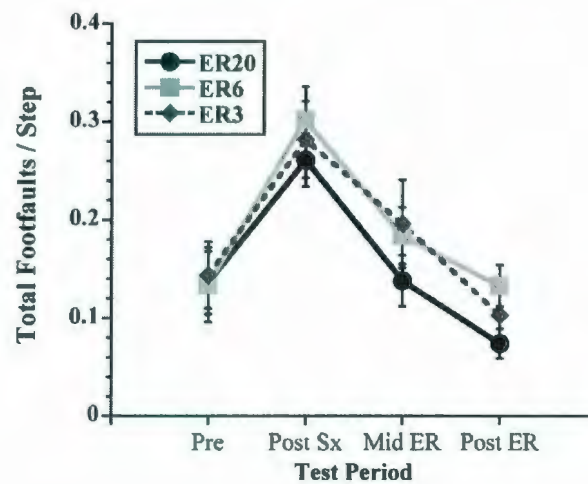


Figure 4.4 Beam-traversing test. All groups exhibited profound impairment in foot placement on day 6 post-ischemia (Post Sx) and showed significant improvements over the treatment period. All groups performed at or near baseline levels by the end of enriched rehabilitation (End ER).

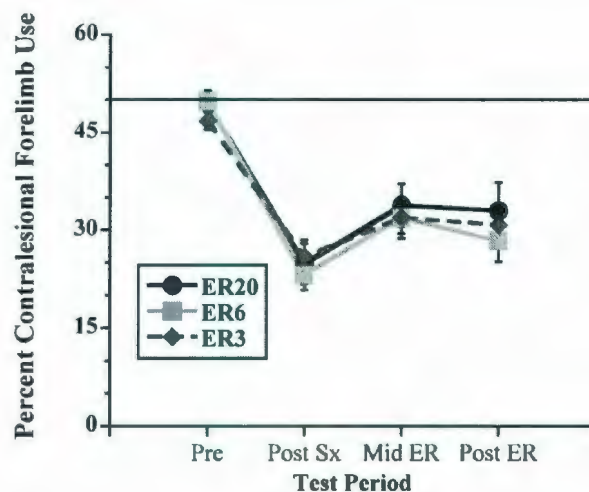


Figure 4.5 Cylinder test of forelimb asymmetry. Animals showed a strong reliance on the ipsilesional forelimb on day 5 post-ischemia (Post Sx). All groups showed a similar but incomplete recovery over the treatment period, continuing to exhibit a notable disuse of the contralesional (i.e. impaired) forelimb at the end of treatment (End ER).

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Chapter 5 Summary

5.1 Summary of main findings

5.1.1 FosB/ Δ FosB immunohistochemistry provides a novel tool for measuring use-dependent neural activation following focal ischemia and treatment

The use of FosB/ Δ FosB immunohistochemistry in Chapter 2 introduced a powerful new method to detect changes in use-dependent neural activity following post-stroke treatment. FosB is a member of the Fos family of transcription factors that, unlike related factors (e.g. c-fos), accumulates only after chronic stimulation (Nestler et al., 1999). This unique feature, along with its prolonged stability, makes it particularly useful to measure neural activation in response to relatively lengthy treatment regimes as opposed to transient perturbations in activity.

FosB/ Δ FosB proteins dimerize with members of the JUN family to form the activator protein-1 complex. Δ FosB has been shown to promote changes in other gene products, including CREB (McClung & Nestler, 2003). Its potential role in the initiation of neural plasticity (McClung et al., 2004) suggests that its use-dependent expression may be a good marker of impending structural or functional adaptation at the cellular level. Indeed, the upregulation of FosB/ Δ FosB by rehabilitative activity may be a key promoter of the

neuroplastic changes that have been associated with functional recovery following longer periods of treatment (Biernaskie and Corbett, 2001).

Previously, activation of neural tissue has been investigated in rodents using intracortical microstimulation (ICMS) (Kleim et al., 1998; Conner et al., 2005) and functional imaging methods (Dijkhuizen et al., 2001). However, neither of these techniques provides a true measure of chronic activity. ICMS, by eliciting muscle movement, determines the somatotopic organization of motor cortex but does not indicate the level of cortical activity in the absence of stimulation. Similarly, while functional imaging is a powerful tool to determine which tissue is activated by specific movement or stimulation it cannot indicate how treatment influences the overall activity within those networks over time. Hence, both methods provide only an endpoint measure of cortical reorganization, but do not directly indicate the processes and mechanisms underlying these changes. In contrast, FosB/ Δ FosB expression is reflective of chronic activation making it particularly useful to measure the recruitment of tissue by rehabilitation and therefore the functional events that may precede structural neuroplasticity and cortical reorganization.

Additionally, FosB/ Δ FosB immunohistochemistry can be used to detect more global perturbations in neural activity following cerebral ischemia or other forms of brain injury. For example, results from Chapter 2 demonstrate that FosB/ Δ FosB expression in the contralesional forelimb motor cortex is suppressed below intact levels in all groups at 9 and 12 days post-ischemia (i.e. after 2 and 5 days treatment). This may be reflective of

widespread metabolic depression and reduced somatosensory circuit activity, referred to as diaschisis, which has been observed transiently following ischemia and traumatic brain injury (Feeney and Baron, 1986; Ginsberg et al., 1989; Buchkremer-Ratzmann et al., 1996). The role of diaschisis in recovery is unclear, and it is possible that the suppression of circuitry may be an integral part of the early reparative process. On the other hand, the resolution of diaschisis has been implicated as a possible mediator in the spontaneous (partial) recovery from impairments that is often observed in the acute phase of stroke (Seitz et al., 1999). Interestingly, the gradual increase in FosB/ Δ FosB expression observed in striatum and contralesional cortex between 2 and 10 days of treatment coincides with a slight improvement in NDS scores in all groups over the same period.

One potential limitation with this method is that it is *ex vivo* and thus requires the animal to be sacrificed in order to carry out analysis, whereas traditional ICMS or functional imaging methods can be used repetitively in living animals. On the other hand, because FosB/ Δ FosB immunohistochemistry can be utilized on brain sections, it allows for direct comparison with other histological measures. For example, in Chapter 2 we were able to assess neuronal activation, delayed cell death, inflammation and infarct volume on the same brains. It would also be possible to investigate the abundance of growth and neurotrophic factors in the same regions as FosB/ Δ FosB expression, determining potential relationships between these post-ischemic events. Development of co-labelling and/or immunofluorescence techniques using FosB/ Δ FosB immunohistochemistry could allow for direct analysis of such relationships at the cellular level. These potential uses

make FosB/ Δ FosB an especially promising tool for elucidating the processes that contribute to neural repair and functional recovery after stroke.

5.1.2 Enriched rehabilitation induces neural activation in intact tissue during the early phase of treatment following focal ischemia

Using the novel technique of FosB/ Δ FosB immunohistochemistry discussed above, we showed in Chapter 2 that enriched rehabilitation enhances neuronal activity in intact perilesional cortex after just 10 days of treatment. This novel finding is important in light of growing evidence that events in the early phase after stroke are conducive to functional recovery, and that rehabilitation is more effective when initiated early. It is likely that this early activation of tissue is a key mechanism underlying the enhanced recovery observed after extended periods of rehabilitation.

Similar trends of enhanced activation in the perilesional striatum and contralesional forelimb motor cortex support notions that both hemispheres may be involved in functional recovery after stroke. Functional recovery following rehabilitation has been associated with cortical reorganization and structural plasticity in both the damaged (Nudo et al., 1996; Conner et al., 2005; Ramanathan et al., 2006) and undamaged hemispheres (Jones and Schallert, 1994; Biernaskie and Corbett, 2001; Biernaskie et al., 2005) following focal ischemia. It is important to note that these changes were observed after significantly longer duration of therapy than that used in Chapter 2, and the enhanced activation we reported after just 10 days treatment likely precedes and possibly mediates these neuroplastic responses.

5.1.3 Enriched rehabilitation promotes functional recovery in a model of focal ischemia targeting forelimb motor cortex and dorsolateral striatum

In Chapter 3, we clearly show that enriched rehabilitation promotes enhanced recovery following a model of focal ischemia that targets forelimb motor cortex and dorsolateral striatum. It has been shown previously that this treatment paradigm produces significant functional recovery following middle cerebral artery occlusion (MCAO) (Biernaskie and Corbett, 2001; Biernaskie et al., 2004). While MCAO models reflect a common form of clinical stroke, the resulting lesion tends to spare forelimb motor cortex (Carmichael, 2005; Windle et al., 2006). Consequently, it is possible that MCAO-lesioned animals are more amenable to rehabilitation of forelimb function and therefore exhibit optimal recovery in tests of skilled reaching and forelimb use. Hence, it was important to replicate the benefits of enriched rehabilitation using another model of focal ischemia that selectively damages cortical structures known to be essential components of forelimb motor control.

Indeed, we showed that following endothelin-1 induced focal ischemia of the forelimb motor cortex and dorsolateral striatum, animals exposed to 9 weeks of enriched rehabilitation showed significantly better functional recovery compared to untreated animals in both the staircase test of skilled reaching and a beam-traversing task. As might be expected, animals exposed to this model of ischemia appeared to show less complete recovery than those exposed to MCAO despite showing similar functional impairments

after surgery. The differing recovery profiles of these (and other) models in response to treatment have implications for choosing appropriate outcome measures in future research.

5.1.4 A periodic return to treatment offers little or no benefit in the chronic phase following focal ischemia

Results from Chapter 3 show that despite significant functional gains following early rehabilitation, a periodic return to therapy did not promote further benefit. Ischemic rats receiving 9 weeks of enriched rehabilitation appeared to plateau and did not improve regardless of whether or not they received additional periods of “tune-up” treatment. This supports evidence that most recovery, both spontaneous and rehabilitation-induced, tends to occur in the first few weeks and that recovery in the chronic phase after stroke is limited (Duncan et al., 1992; Jorgensen et al., 1995).

Additional evidence for this critical time window of recovery is provided by observations that animals that did not receive early rehabilitation showed no improvement when exposed to the later “tune-up” treatment. This suggests that early intervention is important in order to promote functional recovery following stroke, and that delaying treatment for lengthy periods may drastically reduce its effectiveness. Indeed, it has been shown that there is a transient increase in the expression of growth-promoting genes

following ischemia (Carmichael et al., 2005), and delays in rehabilitation may miss this crucial time window when neural repair and recovery is optimally supported (Murphy and Corbett, 2009).

5.1.5 Task specificity and intensity are key components of effective rehabilitation

Importantly, we showed in Chapter 2 that a combination of enriched environment and daily reach therapy, but not enriched environment alone, increased the activity of intact tissue after 10 days treatment. This suggests that task-specific therapy is necessary in order to recruit these uninjured brain regions. Previous studies have demonstrated that reach therapy is required in order to produce significant recovery of skilled reaching in rats exposed to focal ischemia (Biernaskie and Corbett, 2001; Biernaskie et al., 2004). Enriched environment alone has been shown to promote neuroplasticity (Johansson and Belichenko, 2002), neurogenesis (Komitova et al., 2005) and some functional improvement (Ohlsson and Johansson, 1995; Johansson and Ohlsson, 1996) but has proved insufficient to produce recovery of skilled reaching impairments (Grabowski et al., 1993). However, a previous study from our lab has shown that reaching by itself is less effective than when combined with enriched environment, indicating that both are necessary to obtain optimal recovery of skilled reaching function (Pawson et al., 2003).

It is important to note, however, that we did not demonstrate whether reach therapy alone would be sufficient to provide the enhanced neural activity seen following enriched

rehabilitation. This could have been examined using an experimental group that received reach therapy only, in the absence of enriched environment exposure. However, previous experiments in this lab (Pawson et al, 2003) have shown that functional recovery using this approach is minimal and significantly less compared to a combination of daily reaching and environmental enrichment. In light of those findings, and in the interest of conserving animal usage, we did not include a "reach only" group in this set of experiments.

Additional, albeit indirect, evidence for the importance of task specific therapy is provided in Chapter 4 where the daily exposure to enriched environment, but not reach therapy, was varied across treatment groups. Regardless of the amount of enriched environment exposure, all groups showed similar improvements in the staircase test of skilled reaching suggesting that reach therapy may be the more integral component of rehabilitation for the recovery of skilled reaching.

It is interesting to note, however, that animals exposed to "traditional" enriched rehabilitation (i.e. full-time access to enriched environment and daily reach therapy) in this study showed less recovery of skilled reaching function than those in Chapter 3. This difference may be due to the intensity of reaching therapy provided, as those in Chapter 4 received a shorter period of daily access to the reaching apparatus (4 hours versus 6 hours). Evidence from both animal research (Nudo et al., 1996; Kleim and Jones, 2008) and clinical studies (Langhorne et al., 1996; Kwakkel et al., 1997) suggests that intensity

of rehabilitation has a direct impact on the extent of recovery observed following stroke, with greater intensity producing significantly better outcome. Importantly, the number of pellets retrieved during reaching therapy in Chapter 4 correlated with recovery in the staircase test, indicating a direct relationship between rehabilitative activity and functional improvement. These results are supported by the findings of a recent study from our lab indicating that there is a threshold of reaching activity required in order to produce recovery of skilled reaching impairment in ischemic rats. Rats that were allowed to reach a limited number of pellets during reach therapy exhibited less recovery in the staircase test than those that were allowed unlimited reaching during the same period of time (Dr. Crystal MacLellan, pers comm.).

The importance of regular and consistent therapy is further indicated by results in Chapter 2, showing that activation of intact tissue was achieved after 10 consecutive days of therapy but not 5 days. This is important in light of the fact that most rehabilitation centres do not provide weekend therapy. It should be noted, however, that FosB/ Δ FosB is expressed only after chronic stimulation, suggesting that the neural tissue examined was in fact being activated for some time before it was detected using these methods.

5.1.6 Enriched environment has differential influences on the recovery of skilled and unskilled motor function when combined with reaching therapy

Results from Chapter 4 indicate that enriched environment has distinct effects on the recovery of skilled and unskilled motor functions when combined with reaching therapy. All groups, regardless of the extent of daily exposure to enriched environments, showed profound recovery on the beam-traversing task. The persisting impairment in the cylinder task, and evidence from Chapter 3 that untreated ischemic animals show very little recovery on the beam test of foot placement and walking ability, demonstrates that these improvements were due to treatment and not simply spontaneous recovery. While previous studies have shown that continuous enriched environment can promote the recovery of generalized motor functions (Grabowski et al., 1993; Ohlsson and Johansson, 1995; Johansson and Ohlsson, 1996), we are the first to show that as little as 3 hours of daily exposure (combined with reach therapy) is sufficient to produce significant improvement.

On the other hand, enriched environment appears to enhance the rehabilitation of skilled reaching in a dose-dependent manner. Longer durations of exposure, such as that received by animals in the ER20 group, significantly increased the amount of pellets retrieved in the reaching apparatus during the last week of treatment. This improved performance during rehabilitation correlated with greater recovery in the staircase test,

suggesting that more daily exposure to enrichment produced better overall recovery in this task.

Similar results were seen in a previous study that directly compared treatment of rats with enriched environment, daily reach training and a combination of both (i.e. enriched rehabilitation) following MCAO (Pawson et al., 2003). Animals receiving enriched environment or enriched rehabilitation all showed similar recovery in a beam-traversing test, suggesting again that enriched environment promotes recovery of unskilled motor function. Interestingly, animals exposed to enriched rehabilitation exhibited greater recovery in the single-pellet test of skilled reaching than animals receiving either treatment alone, indicating that enriched environment enhances the rehabilitative effect of reach therapy.

5.2 Implications for treatment of stroke

The findings outlined in this thesis have a number of potential implications for stroke patients and the development of novel rehabilitation approaches. Firstly, it is important to develop rehabilitation programs that optimize the recruitment and increased activity of intact tissue, as demonstrated in Chapter 2. Our results suggest that task-specific therapy is an essential component of such rehabilitation, although the selection of activities will likely depend on the location of injury and the primary functional deficits to be targeted. For example, constraint-induced movement therapy has been shown to be effective in promoting enhanced recovery of upper limb impairments (Dean and Shepherd, 1997;

Page et al., 2001), while body-weight supported treadmill therapy is beneficial in patients with gait and walking deficits (Peurala et al., 2005; McCain et al., 2008). In addition to task specificity, it will also be important to determine the optimal timing and intensity of treatment in order to best promote neural activation and functional recovery.

It is possible that adjunct therapies may be useful in enhancing the activation of intact regions in the brain. There is considerable evidence from animal studies indicating that pairing rehabilitation with electrical stimulation of the perilesional motor cortex enhances neuroplasticity and functional outcome (Adkins-Muir and Jones, 2003; Kleim et al., 2003; Plautz et al., 2003; Teskey et al., 2003). This suggests that directly activating neurons near the lesioned motor area during rehabilitation can promote reorganization of motor maps, increased dendritic density, and compensation of motor control. Evidence from clinical studies suggests that combining rehabilitation with similar adjunct therapies, such as transcranial magnetic stimulation, may prove beneficial as a treatment approach (Khedr et al., 2005; Takeuchi et al., 2005; Kim et al., 2006).

Secondly, the increase in FosB/ Δ FosB expression in peri-infarct cortex after 10 days of rehabilitation (Chapter 2) provides new evidence indicating that earlier rehabilitation is better. It is essential that treatment strategies be developed in order to promote as much recovery as possible in the early phase after stroke, because recovery in the chronic phase appears to be limited even with therapy (Nakayama et al., 1994; Jorgensen et al., 1995; Page et al., 2004). Rehabilitation that challenges patients early and becomes

progressively more intense as they recover may prove beneficial, facilitating enhanced recovery rather than allowing for the development of recovery plateaus. However some evidence suggests that intensive, task-specific therapy employed even years after stroke can provide significant benefit for some patients, adding impetus to continued research into more effective late-stage treatments (Dean and Shepherd, 1997; Page et al., 2002; Rijntjes et al., 2009).

Concerns have been raised that early rehabilitation can lead to poorer outcome or recovery after stroke (Kozlowski et al., 1996; Dromerick et al., 2009), however it is likely that these negative effects are due to excessive intensity of rehabilitation at these time points. Lower levels of rehabilitative activity in the early aftermath of stroke have proven beneficial (Kelley and Borazanci, 2009), and treatment plans must take into consideration the potential need for moderation during the most acute phases of recovery.

Thirdly, we have provided additional evidence supporting the notion that complex and stimulating environments need to be incorporated as part of routine stroke rehabilitation. While the value of enriched environments in promoting functional recovery after stroke has been known from animal research for some time, the translation of this principle into clinical practice has been slow (Lincoln et al., 1996; Bernhardt et al., 2004). Specialized stroke units are the closest approximation of enriched environments for humans, and there is strong evidence that they promote better functional outcome than traditional treatment approaches (Teasell et al., 2005). Current lines of evidence from both animal

(Biernaskie and Corbett, 2001; Biernaskie et al., 2004) and clinical (Teasell et al., 2008; Teasell et al., 2009) studies indicate that a combination of intense, task-specific therapy and stimulating environments are important for optimal recovery after stroke, and the results outlined in this thesis provides strong evidence to support those principles.

Stimulating environments for stroke patients should include a variety of activities that promote social, cognitive and physical activity much like "enriched environments" do in the experimental setting. While there are endless possible ways to incorporate stimulating environments, it is important to consider a combination of formal (e.g. exercise programs, social activities and games) and informal (e.g. providing open-access spaces for leisure activity) activities. It may also be advisable to include and promote activities that translate into daily living skills, such as encouraging patients to prepare their own daytime snacks or negotiating around rooms and obstacles that might be encountered outside the hospital setting.

Finally, in addition to promoting maximal recovery in the early treatment stages, efforts must be made to extend the time window when further recovery can be achieved. While our results suggest that a periodic return to therapy does not promote recovery in the chronic phase after stroke, it is possible that more intense and/or targeted rehabilitation may prove more effective. Alternatively, combination therapies (e.g. pharmacological or cell-based therapies combined with rehabilitation) may be developed that facilitate

neuroplasticity and neural repair much later after stroke, making the brain more amenable to recovery in the chronic phase much as it is acutely after injury.

5.3 Future research directions

While the findings outlined in this thesis have documented several principles critical to effective post-stroke rehabilitation and identified novel mechanisms potentially involved in functional recovery, they also open the door on several important avenues for future research.

Firstly, more research is needed to elaborate on the important new findings reported in Chapter 2. While we have presented evidence that the use-dependent activation of intact tissue induced by enriched rehabilitation likely initiates the cortical reorganization and structural plasticity observed at later time points, future studies should aim to confirm this hypothesis. A clear understanding of these earliest effects of rehabilitation and their impact on subsequent neuroplastic responses will help develop optimal strategies to facilitate recovery. Additionally, the results presented in this chapter indicate that the contralesional cortex is recruited during rehabilitation, adding to evidence that it is involved in recovery processes. There is evidence that there is more activation of contralesional cortex in response to larger infarcts (Biernaskie et al., 2005), although the involvement of the intact hemisphere in functional recovery may be attenuated with age (Buga et al., 2008). A better understanding of the specific role that each brain region

plays in functional recovery will help design treatment strategies that can be tailored to individual lesion profiles.

Secondly, while the results presented in Chapter 3 support the notion that the most significant functional recovery may be limited to the early phase after stroke the mechanisms that underlie this time window remain unclear. Building on the findings from Chapter 2, follow-up research should determine if the use-dependent activation of intact tissue observed with early rehabilitation is observed when treatment is delayed. It is known that undamaged areas of the brain are transiently hyperexcitable after cerebral ischemia, and this phenomenon diminishes within days and weeks (Buchkremer-Ratzmann et al., 1996; Neumann Haefelin and Witte, 2000). Consequently, it is possible that the ability of rehabilitation to activate these neural networks declines over the same period and accounts for the limited effectiveness of delayed treatment. Should this be determined, it may be possible to find ways to reinstate this hyperexcitability, thus re-opening the window of neuroplasticity and recovery at later time points.

Thirdly, research at both the basic science and clinical levels should focus on developing rehabilitation approaches that are more effective in the chronic phase of stroke recovery. Our new understanding of the roles that task-specificity, intensity and duration play in the effectiveness of post-stroke rehabilitation, combined with growing knowledge about the molecular mechanisms of neural repair and recovery, should facilitate this line of research. While early post-stroke rehabilitation should be the goal it remains important,

especially for the vast number of patients unable to participate in early rehabilitation, to find ways to optimize late stage recovery.

Finally, an impetus to find new technologies and adjunct treatments to promote recovery at all stages is needed. Technologies such as transcranial magnetic stimulation to enhance neural activation, pharmacological agents to promote neuroplasticity long after stroke onset, and stem/progenitor cell-based therapies to facilitate neural repair hold significant promise for use in the treatment of stroke. The findings outlined in this thesis shed light on both the mechanisms of early rehabilitation and the timeline of functional recovery, providing a basis on which to build a new direction in stroke treatment and research.

5.4 References

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